Old Herborn University Seminar Monograph

4. HOST-MICROFLORA INTERACTIONS IN THE FIRST YEARS AFTER BIRTH

EDITORS:

PETER J. HEIDT
RIAL D. ROLFE
VOLKER RUSCH
DIRK VAN DER WAAIJ

Old Herborn University Seminar Monograph 4

ISBN 3-923022-14-X
ISSN 1431-6579

EDITORS:

Peter J. Heidt, Ph.D., B.M.
Department of Animal Science
Biomedical Primate Research Centre (BPRC)
Lange Kleiweg 139
2288 GJ - Rijswijk
The Netherlands

Rial D. Rolfe, Ph.D.
Department of Microbiology
School of Medicine
Texas Tech University Health Sciences Center
Lubbock, Texas 79430
USA

Volker Rusch, Dr. rer. nat.
Institute for Integrative Biology
Kornmarkt 2
D-35745 Herborn-Dill
Germany

Dirk van der Waaij, M.D., Ph.D.
Professor emeritus, University of Groningen
Hoge Hereweg 50
9756 TJ - Glimmen
The Netherlands

Verlag wissenschaftlicher
Schriften und Bücher
Am Kornmarkt 2
Postfach 1664
D-35745 Herborn-Dill
Germany
Telephone: +49 - 2772 - 921100
Telefax: +49 - 2772 - 921101

Contents

Contents (continued)

Contents (continued)

Participating authors

Patricia M. Bealmear, Ph.D., 65 Brooklea Drive, East Aurora, NY 14052, USA.

Rodney D. Berg, Ph.D., Department of Microbiology and Immunology, Louisiana State University Medical Center, Shreveport, LA 71130, USA.

Mary Boesman-Finkelstein, Ph.D., Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, MO 65212, USA.

Richard A. Finkelstein, M.D., Ph.D., Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, MO 65212, USA.

Lennart Hammarström, M.D., Ph.D., Department of Clinical Immunology and Centre for Biotechnology, Karolinska Institute at Huddinge Hospital, S-14186 Huddinge, Sweden.

Lars Å Hanson, M.D., Ph.D., Department of Clinical Immunology, Göteborg University, Guldhedsgatan 10, S-413 46 Göteborg, Sweden.

David J. Hentges, Ph.D., Department of Microbiology, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA.

Carl Erik Nord, M.D., Ph.D., Department of Microbiology, Huddinge University Hospital, Karolinska Institute, S-141 86 Huddinge, Sweden.

Rial D. Rolfe, Ph.D., Department of Microbiology, School of Medicine, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA.

W. Allan Walker, M.D., Ph.D., Combined Program in Pediatric Gastroenterology and Nutrition at Harvard Medical School, Children's Hospital, 300 Longwood Avenue, Boston, MA 02115, USA.

Old Herborn University Seminar Monograph 4:
Host-microflora interactions in the first years after birth.
Editors: Peter J. Heidt, Rial D. Rolfe, Volker Rusch, and Dirk van der Waaij.
Institute for Microbiology and Biochemistry, Herborn-Dill, Germany: 1-13 (2002).

THE SCID/SCID MOUSE MUTATION: A MODEL FOR THE STUDY OF THE ROLE OF THE MICROFLORA IN THE ONTOGENY OF THE IMMUNE RESPONSE

PATRICIA M. BEALMEAR, DONATO J. BORRILLO, and JOHN J. PAULONIS

Roswell Park Cancer Institute, Buffalo, NY 14263, USA

INTRODUCTION

The SCID mouse mutation, first described by *Bosma* and his colleagues (1983), was derived from a breeding pair of inbred C.B-17 Icr (C.B-17) mice and is a valuable mouse model for studying the ontogeny of the immune system. The C.B-17 mouse is a congenic partner of the BALB/cAnIcr strain, differing from it only by a portion of chromosome 12, that was derived from the C57Ka strain, which carries a recessive gene for diabetes. The Ig heavy chain, found on the same chromosome, and TCR genes have been shown to be present by northern blot analysis, yet, southern blot analysis of SCID/SCID mouse bone marrow and foetal liver hybridomas showed no Ig heavy chain rearrangement (*Schuler* and *Bosma*, 1989; *Schuler* et al., 1990). The mutant locus implicated in immunodeficiency followed autosomal recessive gene control, was mapped close to mahoganoid and centromeric to the Ig lambda light chain locus on chromosome 16 and had low frequency of Ig gene and T-cell receptor gene rearrangements (*Bosma*, 1989; *Bosma* et al., 1983, 1989; *Schuler* and *Bosma*, 1989). This new strain was deficient in all major immunoglobulin classes and T-cell activity, with the ability to accept allografts and/or xenografts. Myeloid and erythroid lineages are apparently unaffected by the mutation, with near normal numbers of spleen colony forming units (CFU-S) and granulocyte-macrophage colony forming units (GM-CRU) (*Phillips* and *Fulop*, 1989). Macrophage and natural killer (NK) spleen cell activity are normal (*Kumar* et al., 1989). Macrophage activation can occur in a T cell-independent manner and may constitute an important model to unravel the mechanism of 'natural' resistance to infection (*Ansell* and *Bancroft*, 1989; *McCune* et al., 1988).

Penetrance of this mutation is not complete (*Caroll* et al., 1989) with 41% of SCID/SCID mice older than 9 months and 15% of younger SCID/SCID mice producing a limited clonal diversity of serum Ig as shown by isoelectric focusing (*Gibson* et al., 1989). These mice are termed 'leaky' mice; the degree of 'leakiness' is directly related to the number and kind of contaminants in the microflora, therefore, this animal is a good model for demonstrating the role of the microflora in the ontogeny of the immune response.

Nishikawa and colleagues (1989) have shown *in vitro* that the pluripotent stem cells are committed to produce lymphoid cells; their defect appears prior to expression of cytoplasmic or surface immunoglobulin (supporting chromosomal defect) with arrest in the pre-pre-B stage, thus, Ig-cells bearing Thy-1 and low levels of the leukocyte common antigen B220 can be detected in the spleen and bone marrow, although they are considerably reduced in number (*Hardy* et al., 1989; *Nishikawa*

et al., 1989).

While pre-B cells are undetectable in SCID/SCID tissues, they can be generated from SCID/SCID bone marrow by long-term Witlock-Witte/Dexter culture methods in the presence of IL-7 (*Lee* et al., 1989; *Nishikawa* et al., 1989). The frequency of responding cells and the expansion potential of pre-B colonies produced by these methods is severely limited, and they have limited survival (*Nishikawa* et al., 1989). Since SCID/SCID mouse stromal cells are able to support bone marrow and foetal liver transplants, a micro-environmental defect that suppresses B-cell differentiation is ruled out (*Nishikawa* et al., 1989).

T-cell development in SCID/SCID mice arrests at a point equivalent to 14-15 days of gestational age (C.B-17 control) with the majority of SCID/SCID thymocytes expressing double negative CD4 and CD8 with positive Thy-1 (*Habu* et al., 1989). Forty to sixty percent express interleukin 2 (IL-2) receptors and will divide in response to recombinant IL-2 (*Hardy* et al., 1989; *Nishikawa* et al., 1989). *Shores* et al. (1990) introduced normal bone marrow cells into TcR^- SCID mice and these gave rise to TcR^+ cells within the SCID thymus and promoted the differentiation of SCID thymocytes into $CD4^-CD8^+$ and $CD4^+CD8^+$ TcR^- cells.

Kumar and colleagues (1989) demonstrated that natural killer cell differentiation in the SCID/SCID mouse spleen cell population is unaffected by the mutation with normal numbers of NK progenitors in the marrow giving rise to functional $NK2.1^+$ $ASG1^+$ cytotoxic cells which do not express T-cell markers. Mature NK cells, but not their progenitors have been detected in SCID/SCID spleen. It was concluded that either NK cells were derived from T cells or they diverged from a common progenitor in the marrow prior to the expression of the SCID/SCID phenotype (*Kumar* et al., 1989).

Garni-Wagner et al. (1990) investigated the relationship between NK cell and T-cell progenitors using the thymus of SCID/SCID mice. Two populations of cells have been identified in the hypocellular SCID/SCID mouse thymus. Eighty percent of the cells are $Thy\text{-}1^+$, $IL\text{-}2R(7D4)^+$, $J11d^+$ (T progenitors), $CD3^-$, $CD4^-$, $CD8^-$, and twenty percent of the cells are $IL\text{-}2R^-$, $J11d^-$, $CD3^-$, $CD4^-$, and $CD8^-$; NK activity is found in the second population, which is phenotypically similar to splenic NK cells. Cultured $J11d^+$ thymocytes acquired non-MHC-restricted cytotoxicity, but differed from mature NK cells by containing mRNA for the γ, δ, and ε-chains of CD3. This suggests that $J11d^+$ cells are early T cells that can acquire cytotoxic potential for non-MHC-restricted cells, but they do not give rise to NK cells *in vitro*. *Garni-Wagner* et al. (1990) suggest that mature NK cells reside in the SCID/SCID mouse thymus, but they are not derived from a common NK/T progenitor.

SCID/SCID genotype

Normal murine germline rearrangement results in the generation of immune diversity with antigen specific antibody (Ig), thymocyte cell receptors (TCR), and major histocompatibility complexes (MHC). Antigen is recognised by the variable domains of the Ig molecule (*Ward* et al., 1989); diversity in the variable domain of the heavy chain is achieved somatically by the joining of three gene segments, VH (variable), DH (diversity) and JH (joining) (*Hozumi* et al., 1976; *Kurosawa* et al., 1981). The VH segment consists of two exons, one that encodes most of the leader peptide and which is not pre-

Table 1: Genome of mouse and man (*Lewin*, 1985)

Family	Located on chromosome		Number of V genes		Number of C genes		% chain type	
	Human	Mouse	Human	Mouse	Human	Mouse	Human	Mouse
Lambda	2	16	~300	2	>6	3	40	5
Kappa	22	6	~300	~300	1	1	60	95
Heavy	14	12	~300	>100	9	8	100	100

the leader peptide and the first 95 amino acids of the variable domain.DH is a small gene segment encoding about 3-8 amino acids of the third hypervariable region and all of framework 4. In addition N sequences, i.e., nucleotides that can be added to the boundaries of the gene segments during V(D)J joining, can be present (*Wu* et al., 1990). VH gene usage by mouse and human has an early bias at the 3' end of the array of VH segments; the closer a gene segment is to the (D)J structure, the more likely it is to recombine (*Yancopoulos* et al., 1984); this bias may reflect the functioning of these gene products early in ontogeny (*Wu* et a., 1990). This biased usage is strain dependent, notably in the BALB/c strain from which the C.B-17 mouse strain was derived.

Variable region genes are assembled during the antigen-independent phase of B cell differentiation (*Malynn* et al., 1990). This occurs in primary B cell differentiation organs, e.g., foetal and neonatal liver and bone marrow. Pre-B lymphocytes assemble and express heavy and light chain genes to become surface Ig+ B cells; this is the "newly generated" antibody repertoire unselected by external antigens (*Malynn* et al., 1990).

The mouse immunoglobulin protein is composed of two identical heavy chains and two identical light chains. All heavy chain genes are found on germline chromosome 12 and are arranged linearly of greater than 100 variable genes, approximately 10 diversity genes, 4 joining segment genes, and 8 constant genes (*Lewin*, 1985). In contrast, light chain production may be either kappa (κ), found on germline chromosome 6, or lambda (λ), found on germline chromosome 16. Only 5% of light chain production is of lambda origin (Table 1), with the majority being kappa (95%). The kappa germline gene linearly is composed of approximately 300 variable genes, 5 joining segment genes, and 1 constant gene; the lambda germline gene is composed of 2 variable genes, 1 joining gene, and 3 constant genes (*Lewin*, 1985).

All murine somatic cells contain the above germline genes, which, undergo somatic recombination in lymphocytes to produce immature B cells with specific antibody diversity. Upon antigenic stimulation, one specific antibody-presenting immature B cell will proliferate to secrete antibody or remain dormant as a memory cell (*Lewin*, 1985).

Recombinase activity can be conferred to 3T3 cells, via transfection, using SCID/SCID DNA as a source of the recombinase-activating gene (Rag-1 element). Recombinational activity for exogenous plasmid substrates is conferred, but whether this SCID/SCID RAG-1 element confers normal or abnormal recombinase activity has not yet been determined (*Weaver*, 1989). Thus, inability of these mice to join

coding regions of V, D, and J heavy segments provides a sufficient explanation for the absence of T and B cells in the mutant (*Weaver*, 1989). *Schuler* et al. (1990) reported a high frequency of abnormal Igh and TcR β gene rearrangements in transformed immature SCID lymphoid cells, which typically involved large J segment-associated deletions resulting from attempted D-J recombination.

Normal murine T cells have a variety of functions connected with interactions between cells involved in the immune response. T-cell function involves production of the T-cell receptor (TCR), a set of transmembrane glycoproteins, that provide a direct counterpart to the antibodies produced by B cells. The TCR must recognise a foreign antigen of unpredictable structure and recognise histocompatibility (*Lewin*, 1985; *Carbonari* et al., 1990).

The TCR is actually a complex (TCR/CD3), and is made of either alpha-beta (α-β) chain TCR or gamma-delta (γ-δ) chain TCR, both associated with a constant CD3 element. Alpha-beta chain TCR is found predominantly on peripheral blood T cells and central lymphoid organ T cells. Gamma-delta TCR is present on an immature minor population of cells, predominantly in bone marrow. During intrathymic differentiation, genes are first expressed for the CD3 proteins and then the TCR, however, the TCR/CD3 complex will not appear on the cell surface if either TCR alpha or beta chain is absent. Transfection of TCR alpha or beta genes into mutant cells deficient in synthesis will restore surface expression, therefore, it is thought that one of the TCR genes is a limiting determinant (*Carsten* et al., 1989).

In adult thymocytes from SCID/SCID mice, TCR alpha, beta and gamma genes are in the germline configuration with the presence of beta and gamma transcripts. Examination of the delta locus showed a restricted number of sub-germline bands consistent with attempted diversity-delta-2 to joining-delta-1 rearrangement. This confirms that there may be an ordering of TCR recombinational events during T-lymphocyte differentiation, with delta rearrangement occurring first and representing a selective advantage for this recombination (*Caroll* and *Bosma*, 1989).

Rescue of the SCID/SCID mouse immune system by transgenic introduction of productively rearranged Ig genes has resulted, to a limited extent, in B-cell maturation to IgM synthesis (*Fried* et al., 1989). Alpha and beta TCR chain transgenic introduction has rescued SCID/SCID thymocytes to $CD4^+/CD8^+$ maturation, but further proliferation and maturation occurred only in transgenic mice expressing MHC. This shows the importance of appropriate thymic MHC-TCR interaction for T-cell development (*von Boehmer* and *Blüthmann*, 1989).

Croitoru et al. (1990) identified intraepithelial leukocytes (IEL) in SCID/SCID mice lacked CD3 expression and mRNA for the V.7 V region gene of the T cell receptor. They concluded that these IEL differ from classical T cells in their ability to differentiate and express CD8 and do not require T cell receptor expression for their localisation to the intestine.

'Leaky' SCID/SCID mice

"Leakiness" in the SCID/SCID mouse refers to the somatic expression of immunoglobulin (Ig) by a SCID/SCID mouse population as it ages under non-specific pathogen-free conditions. This may indicate that the penetrance of this mutation is not complete (*Caroll* et al., 1989). Forty-one percent of SCID/SCID mice older than 9 months and 15% of younger

SCID/SCID mice produce a limited clonal diversity of serum Ig as shown by isoelectric focusing (*Gibson* et al., 1989). An oligoclonal pattern usually of between 1 and 12 clonotypes is seen with little sequential variation in these patterns, thereby, suggesting that the leaky phenotype occur at the level of B-cell precursor. Since this is prior to VDJ rearrangement, B cells have little subsequent potential for expansion and differentiation (*Gibson* et al., 1989).

Leakiness is strongly determined by reactivity to autoantigens as shown by B-cell hybridomas generated from spleens of leaky SCID/SCID mice, which are specific for host cell nuclei, erythrocytes and platelets as well as to the enteric pathogens of *Enterobacter* or *Serratia* origin (*Kearney* et al., 1989).

At the molecular level, limited differentiation of T lymphocytes can be shown in leaky SCID/SCID mice. When spleen cells from leaky SCID/SCID mice are cultured *in vitro* and probed for TCR expression they show the expansion of only 1-5 clones per spleen, but the majority of cells from these clones have apparently normal TCR gene rearrangements (*Carroll* et al., 1989).

Injection of purified B-cell hybridoma antibody into neonatal SCID/SCID mice induces both T- and B-cell development. The T-cell population has been said to expand with Ig production as a stimulus; $CD3^+$ cells are present ($<10^5$) and skin grafts can be rejected, but the CD4/CD8 ratio remains skewed (*Kearney* et al., 1989). This illustrates the co-ordinate development of both cell lineages in the leaky phenomenon, and when taken together the data for T- and B-cell differentiation in leaky SCID/SCID mice favours an epigenetically driven reversion of mutational events rather than a stochastic process of chance productive gene rearrangements (*Ansell* and *Bancroft*, 1989). The question is what role the microflora and/or its products play in this reversion.

MATERIAL AND METHODS

Animals

Inbred SCID/SCID mice were obtained from the Johns Hopkins University Oncology Center Animal Resources Division, Baltimore, MD, USA; the original SCID/SCID mice came from M.J. Bosma, Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA, USA. Currently they are housed in micro-isolators, but are being caesarean-derived and placed into the germfree environment.

Inbred germfree DBA/2Wg mice, originally obtained from Walberg, Oak Ridge National Laboratory in l969; inbred germfree C3H/HeSchGN mice, originally obtained from the A.R. Schmidt Co. (now Harlan Sprague Dawley, Inc.); inbred BALB/c nu/nu mice, originally obtained from the Department of Radiation Medicine, Massachusetts General Hospital, are maintained by brother-sister matings in flexible plastic film isolators and treated according to the standard procedures in use at the LOBUND Laboratory for germfree mice (*Trexler*, 1959; *Wagner*, 1959).

All mice are free of *Pneumocystis carinii* and of all murine viruses, with the exception of latent leukaemia virus, which is carried by all strains of germfree mice. Sacrifice was by cervical

Table 2: Fasting glucose levels of SCID/SCID C.B-17 mice

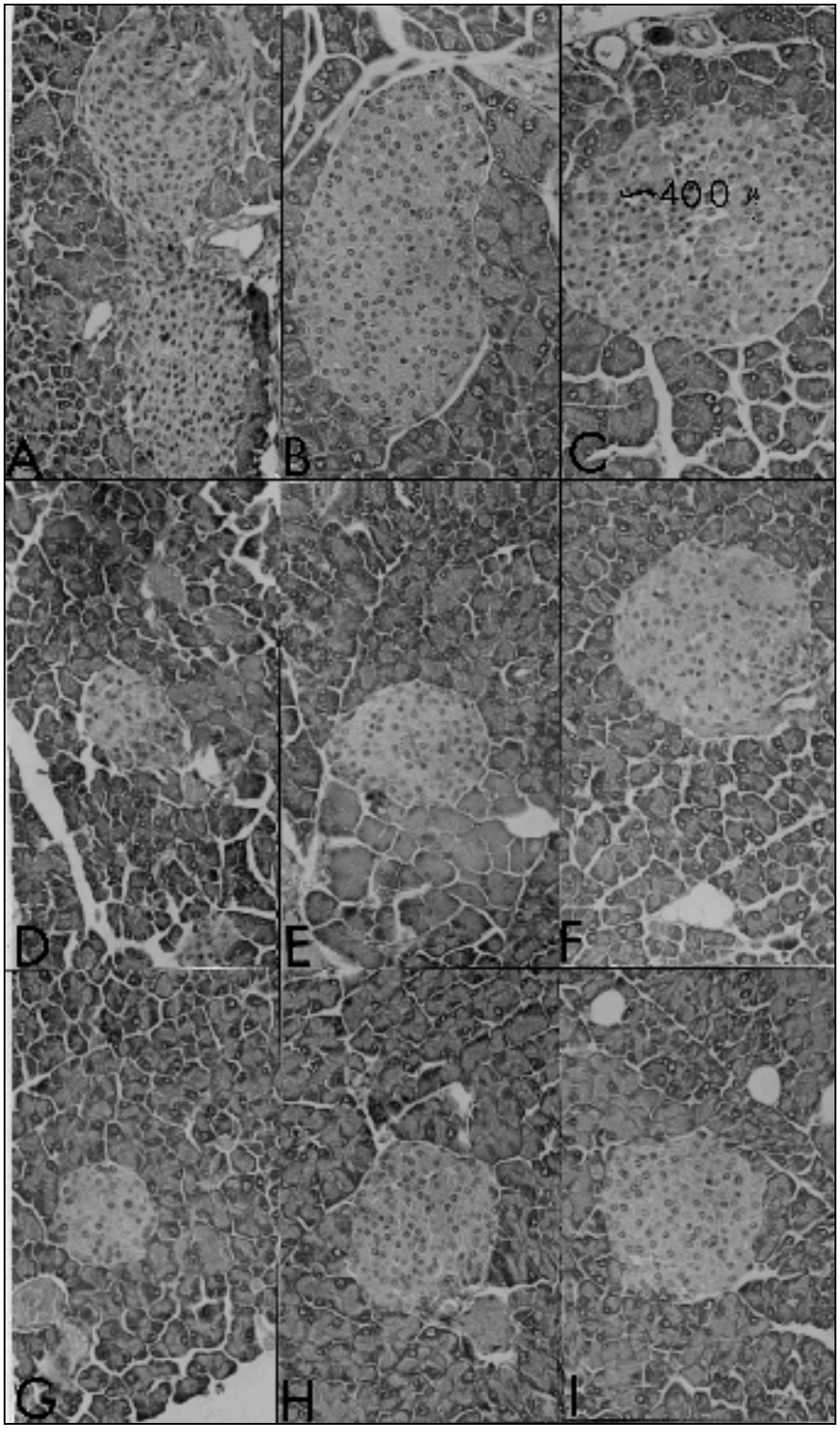
Strain	Birth date	Age	Blood glucose (mg/100 ml)
SCID/SCID	09/04/89	26 wk	52 <40
C.B-17	10/30/89	17 wk	152 147
CFW/Bel	12/17/89	$10^{1}/_{2}$ wk	112 130
C3H/HeNSch	01/25/90	5 wk	92 112
DBA/2Wg	08/27/89	26 wk	92 93
C57Bl/6J	01/25/90	5 wk	120 130
BALB/c nu/nu	08/21/89	27 wk	80 100

dislocation; tissue specimens were removed, placed into Bouin's fixative for 1 hr, then placed into 70% EtOH until paraffin embedding and sectioning at 5 mm. Slides were either stained with routine haematoxylin and eosin or with Gomori stain, which permitted the separation and identification of alpha and beta islet cells in the pancreas.

Blood glucose monitoring

Blood glucose levels were sampled by retro-orbital bleeding (Riley, 1960) via a haematocrit capillary tube, with a minimum of two. Mice were under plane II ether anaesthesia for this procedure. Blood samples were tested by a Tracer II blood glucose monitor and test strips (Boehringer Mannheim Biochemicals, Indianapolis, IN 46250, USA), and are reported in mg/dl by digital display. Preliminary studies included two mice of each strain as described in the results (Table 2); the confirmative study included 8 male SCID/SCID adult mice and 5 female SCID/SCID adult mice; controls were 7 male and 5 female C.B-17 adult mice. All mice were sampled in the morning between 7 and 10 a.m. after an overnight 12 hr fast; water was supplied *ad libitum*.

Figure 1: SCID/SCID male pancreas (A); SCID/SCID female pancreas (B); pregnant SCID/SCID female pancreas (C). Normal exocrine structure; hyperplastic (est.~400 mm) islet of Langerhans; elliptically-shaped islet of Langerhans in (B); endocrine cellular morphology normal. Balb/c nu/+ male pancreas (D); Balb/c nu/+ female pancreas (E); Balb/c nu/+ pregnant female pancreas (F). Normal exocrine structure; islet of Langerhans slightly enlarged in pregnant female (F), ~225 mm; DBA/2Wg male pancreas (G); DBA/2Wg female pancreas (H); DBA/2Wg pregnant female pancreas (I). Fasting state serous cells; small sized islet of Langerhans (est. 175 mm).
All tissues are stained with H & E. (215x)


A
B
C
400 μ
D
E
F
G
H
I

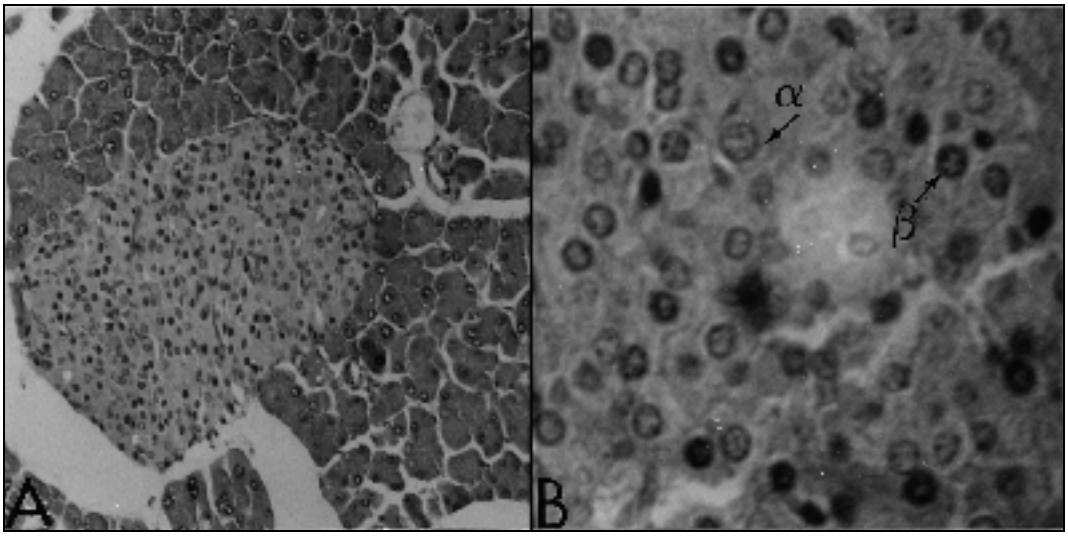


Figure 2: Photomicrograph of SCID/SCID mouse pancreas. Photomicrograph of adult female pancreas stained with trichrome stain. Alpha cells are hyperplastic.
A. 240x; B. 480x.

RESULTS

SCID/SCID mouse lymphoid follicles in the spleen, mesenteric and popliteal lymph nodes and the 'medullary' thymus consisted of stromal cells, histiocytes, and granulocytes and were devoid of lymphocytes (*Bealmear* et al., 1990). Erythroid hyperplasia and some megakaryocytosis were observed in the red pulp of the spleen. Haematocrits were normal and peripheral blood and bone marrow were characterised by 'leukocytosis', lymphopenia. Peyer's patches were sparse and devoid of lymphoid cells. Hepatic, lung, and renal parenchyma were normal; cardiovascular architecture had no congenital defects or myopathy. Adrenal cortex and medulla appeared hypertrophic and warrant further investigation of the juxta-medullary x-zone layer (*Arey*, 1963). The ovaries had follicles and eggs in all stages of development, however the testicles appeared to have few sperm. Skeletal muscle and central nervous system were normal.

The pancreas was grossly of normal size and consistency for all strains taken in the study; no masses, nodularity, or sites of ectopic (i.e., metastatic) tissue were found. No gross systemic disease was noted; all mice were considered to be in a general state of good health.

Exocrine and endocrine components of the pancreas were examined microscopically. The exocrine component of all strains was examined for congenital duct anomaly, signs of regressive changes (i.e., fatty infiltration, atrophy, etc.), inflammatory processes (i.e., acute or chronic pancreatitis), and tumours (both cystic and carcinomatous). None of the abnormalities listed were observed. A non-uniform staining of serous cytoplasm was noted in C3H/HeNSch female, and DBA/2Wg pregnant female sections (*Borrillo* and *Bealmear*, 1990). This was attributed to normal physiological changes in the digestive phase of these mice.

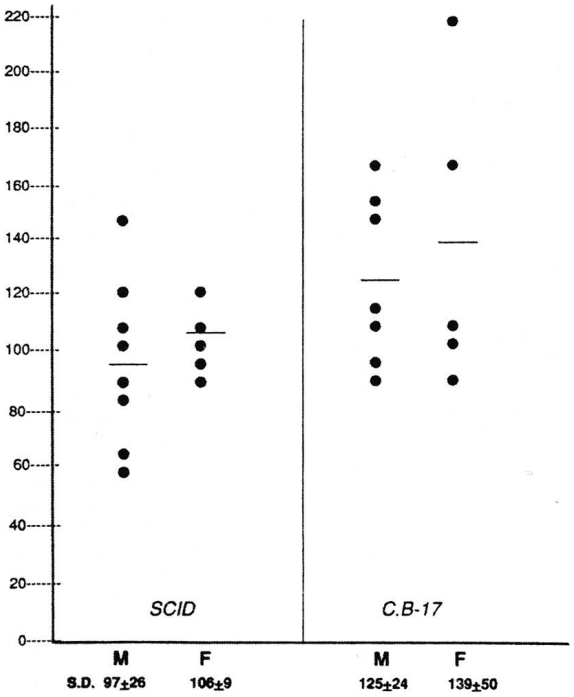


Figure 3: Fasting blood glucose levels (mg/dl) of SCID/SCID vs. C.B-17 Mice. All mice were older than 12 wk of age. Female mice were nongravid.

All microscopic examination of at least 50 slides from pancreas sections from SCID/SCID mice, stained with haematoxylin and eosin, revealed islets which were either round or oval in shape, and averaged >400 mm (Figure 1). No inflammation or fibrosis of the islets or surrounding exocrine pancreas was noted. There was no evidence of hyalinisation or dysplasia and excess mitotic activity was not evident.

Examination of liver sections did not show evidence of ectopic islet cells. Gomori and Masson staining of SCID/SCID mouse pancreas (Figure 2) demonstrated an abnormal central abundance of pink staining alpha (glucagon-producing) cells mixed within the normally predominating beta (insulin-producing) cells, which stain blue.

Small and medium-sized islets from all the other strains examined (seven strains representing male and female (both pregnant and nonpregnant) were identical in appearance to those of the SCID/SCID mouse, except for their diameter, which averaged <300 mm. Pancreatic sections from all controls showed a predominance of small islets. However, since large and medium-sized islets comprise most of the total volume, subsequent estimation of islet size included only those islets with diameters of at least 100 mm.

Preliminary male SCID/SCID fasting blood glucose levels (Table 2) were decreased in comparison to all strains and ages sampled (*Borrillo* and *Bealmear*, 1990), a hypoglycaemic state not seen during nonfasting. Female fasting blood glucose levels showed no trend. This original study was confirmed in a larger sampling of 13 SCID/SCID mice and their C.B-17 controls (Figure 3).

DISCUSSION

The islet hyperplasia may be age dependent, because as mice age, the growth rate of islets tends to accelerate. In the Wellesley rat strain, which tends to be obese, 50% of the males and 5% of the females become glycosuric between the ages of 16 and 55 weeks with nearly all exhibiting extreme islet cell hyperplasia between 12-30 months (*Jones*, 1964). Obese hyperglycaemic V strain mice, as a result of their Mendelian recessive transmission, allow the study of lean and obese litters. Using this strain, *Bleisch* et al., (1952) showed the islets of Langerhans to be hyperplastic predominantly in obese mice (i.e., 50-60 g at 12 months) (*Black* et al., 1988). Although not obese, the SCID/SCID mouse does maintain its hyperplasia with age, but does not have an associated hyperglycaemia or the characteristic diabetic lesions, i.e., beta cell degranulation with vacuolisation, islet hyalinisation, or leukocyte infiltration. Rather, an association between immune function and islet size should be considered because of the nature of the SCID/SCID defect. We postulate that the immune system may play a role in suppression of normal long-term islet cell growth, a role that should not always be viewed as an 'autoimmune pathology'. An overexpression of this suppressive role may lead to diabetes, just as an undersuppression may lead to hyperplasia or malignancy. In this model, the short term regulation of islet cells would still be under blood glucose control, and in the SCID/SCID mouse could account for the alpha cell hyperplasia; an imbalance among the cells of the immune system may be responsible for the suppression of islet-cell glucose receptor antibody and upset the delicate balance between glucose and insulin. Other endocrine glands, their secretions, and their synergistic effect(s) on the immune system should be studied before the severe combined immune deficiency defect can be clearly defined.

LITERATURE

Ansell, J.D., and Bancroft, G.J.: The biology of the scid mutation. Immunology Today 10, 322-325 (1989).

Arey, L.B.: Human histology. W.B. Saunders Co., Philadelphia (1963).

Bealmear, P.M., Borrillo, D.J., Paulonis, J.J., Valenti, J.S., Mascaro, F.J., and Becker, C.J.: Comparative histology of the scid/scid mouse versus C.B-17, Balb/c +/+ & nu/nu, and C3H/HeNSch mice. In: Experimental and clinical gnotobiology. Proceedings of the 10th International Symposium on Gnotobiology (Eds.: Heidt, P.J., Vossen, J.M., and Rusch, V.C.). Microecol. Ther. 20, 271-278 (1990).

Black, M.A., Heick, H.M., and B'egin-Heick, N.: Abnormal regulation of cAMP accumulation in pancreatic islets of obese mice. Amer. J. Physiol. 255, 833-838 (1988).

Bleisch, V.R., Mayer, J., and Dickie, M.M.: Familial diabetes mellitus in mice, associated with insulin resistance, obesity and hyperplasia of the islands of Langerhans. Amer. J. Pathol. 28, 369-385 (1952).

Borrillo, D.J., and Bealmear, P.M.: Hyperplastic islets of Langerhans in the pregnant nonobese scid mouse. In: Experimental and clinical gnotobiology. Proceedings of the 10th International Symposium on Gnotobiology (Eds.: Heidt, P.J., Vossen, J.M., and Rusch, V.C.). Microecol. Ther. 20, 295-303 (1990).

Bosma, G.C., Custer, R.P., and Bosma, M.J.: A severe combined immune deficiency muta-

tion in the mouse. Nature 301, 527-530 (1983).

Bosma, M.J.: The scid mutation: occurrence and effect. In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 1-9 (1989).

Bosma, G.C., Davisson, M.T., Reutsch, N.R., Sweet, H.O., Schultz, L.D., and Bosma, M.J.: The mouse mutation severe combined immune deficiency (scid) is on chromosome 16. Immunogenetics 29, 54-57 (1989).

Carbonari, M., Cherchi, M., Paganelli, R., Giannisi, G., Galli, E., Gaetano, C., Papetti, C., and Fiorilli, M.: Relative increase of T cells expressing the gamma/delta rather than the alpha/beta receptor in ataxia-telangiectasia. New Eng. J. Med. 322, 73-76 (1990).

Caroll, A.M., Hardy, R.R., Petrini, J., and Bosma, M.J.: T cell leakiness in scid mice. In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 117-123 (1989).

Caroll, A.M., and Bosma, M.J.: Rearrangement of T cell receptor delta genes in thymus of scid mice. In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 63-67 (1989).

Clevers, H., Alarcon, B., Wileman, T., and Terhorst, C.: The T cell reseptor/CD3 complex: a dynamic protein ensemble. Ann. Rev. Immunol. 6, 629-640 (1988).

Croitoru, K., Stead, R.H., Bienestock, J., Fulop, G., Harnish, D.G., Shultz, L.D., Jeffery, P.K., and Ernst, P.B.: Presence of intestinal intraepithelial lymphocytes in mice with severe combined immunodeficiency disease. Eur. J. Immunol. 20, 645-651 (1990).

Fried, M., Hardy, R.R., and Bosma, M.J.: Transgenic scid mice with a functionally rearranged immunoglobulin heavy chain gene. In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 107-114 (1989).

Garni-Wagner, B.A., Witte, P.L., Tutt, M.M., Kuziel, W.A., Tucker, P.W., Bennett, M., and Kumar, V.: Natural killer cells in the thymus: studies in mice with severe combined immune deficiency. J. Immunol. 144, 796-803 (1990).

Gibson, D.M., Bosma, G.C., and Bosma, M.J.: Limited clonal diversity of serum immunoglobulin in leaky scid mice. In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 125-136 (1989).

Habu, S., Norihisa, Y., Sato, T., Yagita, H., and Okumara, K.: Phenotype and differentition stage of scid mouse thymocytes. In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 27-31 (1989).

Hardy, R.R., Kemp, J.D., and Hayakawa, K.: Analysis of lymphoid population in scid mice: detection of a potential B lymphocyte progenitor population present at normal levels in scid mice by three color flow cytometry with B220 and S7. In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 19-25 (1989).

Hozumi, N., and Tonegawa, S.: Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. Proc.Natl. Acad. Sci. 73, 3628-3632 (1976).

Jones, E.E.: Spontaneous hyperplasia of the pancreatic islets associated with glycosuria in hybrid mice. In: The structure and metabolism of the pancreatic islets (Eds.: Brolin, S.E., Hellman, B., and Knutson, H.). Pergamon Press, New York, 189-191 (1964).

Kearney, J.F., Solvason, N.W., Stohrer, R., Ma, J., van Cleave, V., Heun, A., Fulop,

G.M., and Fried, M.: Pauciclonal B cell involvement in production of immunoglobulin in scid Ig+ mice. In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 137-147 (1989).

Kumar, V., Hackett Jr., J., Tutt, M.M., Garni-Wagner, B.A., Kuziel, W.A., Tucker, P.W., and Bennett, M.: Natural killer cells and their precursors in mice with severe combined immunodeficiency. In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 47-51 (1989).

Kurosawa, Y., von Boehmer, H., Haas, W., Sakano, H., Trauneker, A., and Tonegawa, W.S.: Identification of D segments of immunoglobulin heavy-chain genes and their rearrangement in T lymphocytes. Nature 290, 565-570 (1981).

Lee, G., Medina, K., and Kincade, P.W.: Growth requirements of B lineage lymphocytes from scid and normal mice. In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 33-37 (1989).

Lewin, B.: Genes. 2nd Ed. John Wiley & Sons, New York (1985).

Malynn, B.A., Yancopoulos, G.D., Barth, G.D., Bona, C.A., and Alt, F.W.: Biased expression of JHproximal VH genes occurs in the newly generated repertoire of neonatal and adult mice. J. Exp. Med. 17, 843-859 (1990).

McCune, J.M., Namikawa, R., Kaneshima, H., Shultz, L.D., Liebermann, N., and Weissman, IL.: The scid-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. Science 241: 1632-1639 (1988).

Nishikawa, S.I., Hayashi, S.I., Nishikawa, S., Ogawa, M., Kunisada, T., Sodo, T., Kodama, H., and Suda, T.: In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 39-45 (1989).

Phillips, R.A., and Fulop, G.M.: Pleiotropic effects of the scid mutation: effects on lymphoid differentiation and on repair of radiation damage. In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 11-17 (1989).

Riley, V.: Adaptation of orbital bleeding technic to rapid serial blood studies. Proc. Soc. Exp. Biol. Med. 104, 751-754 (1960).

Schuler, W., and Bosma, M.J.: Nature of the scid defect: a defective VDJ recombinase system. In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 55-61 (1989).

Schuler, W., Schuler, A., and Bosma, M.J.: Defective V-to-J recombination of T cell receptor chain genes in scid mice. Eur. J. Immunol. 20, 545-550 (1990).

Shores, E.W., Sharrow, S.O., Uppenkamp, I., and Singer, A.: T cell receptor-negative thymocytes from SCID mice can be induced to enter the CD4/CD8 differentiation pathway. Eur. J. Immunol. 2, 69-77 (1990).

Trexler, P.C.: The use of plastics in the design of isolator systems. Ann. N.Y. Acad. Sci. 78, 29-36 (1959).

von Boehmer, H., Blüthmann, H., Teh, H.S., and Scott, B.: The utilization of the scid mutation in the study of T cell development. In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 97-105 (1989).

von Boehmer, H.: Developmental biology of T cells in T cell receptor transgenic mice. Ann. Rev. Immunol. 8, 531-545 (1990).

Wagner, M.: Serological aspects of germfree life. Ann. N.Y. Acad. Sci. 78, 261-271 (1959).

Ward, E.S., Gussow, D., Griffiths, A.D., Jones, P.T., and Winter, G.: Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli.

Nature 341, 544-546 (1989).
Weaver, D., and Hendrickson, E.: The scid mutation disrupts gene rearrangement at the rejoining of coding strands.In: Current topics in microbiology and immunology: The scid mouse (Eds.: Bosma, M.J., Phillips, R.A., and Schuler, M.). Springer-Verlag, New York, 77-83 (1989).
Wu, G.E., Atkinson, M.J., Ramsden, D.A., and Paige, C.J.: VH gene repertoire. Sem. in Immunol. 2, 207-216 (1990).
Yancopoulos, G.D., Desiderio, S.V., Pasking, M., Kearney, J.F., Baltimore, D., and Alt, F.W.: Preferential utilization of the most JH-proximal VH gene segments in pre-B-cell lines. Nature 311, 727-733 (1984).

Old Herborn University Seminar Monograph 4:
Host-microflora interactions in the first years after birth.
Editors: Peter J. Heidt, Rial D. Rolfe, Volker Rusch, and Dirk van der Waaij.
Institute for Microbiology and Biochemistry, Herborn-Dill, Germany: 14-21 (2002).

THE HUMAN INTESTINAL MICROFLORA DURING THE FIRST YEAR OF LIFE

RUTGER BENNET[1] and CARL ERIK NORD[2]

[1]Department of Paediatrics, St. Görans Hospital, S-112 81 Stockholm, Sweden, and [2]Department of Microbiology, Huddinge University Hospital, Karolinska Institute, S-141 86 Huddinge, Sweden.

INTRODUCTION

The extensive development of neonatal intensive care has produced a new, surviving population of extremely vulnerable immature infants, adapted to the sterile amniotic fluid, and not to an environment replete with bacteria. Many of them, in addition to receiving broad-spectrum antibiotics, are neither healthy, nor vaginally delivered, nor breast-fed, nor full-term.

In this article, an overview of the initial colonisation and the development of the microflora during the first weeks of life, especially in new-born infants subjected to intensive care management, is given. The consequences of microbial colonisation for these infants are also discussed.

NORMAL DEVELOPMENT OF INTESTINAL MICROFLORA

The normally sterile foetus encounters a "hodgepodge" of microorganisms at the moment of rupture of the foetal membranes. In the study by *Brook* et al. (1979), the microflora of gastric contents of a mere 5-10 minutes old baby was found to reflect the cervical flora of the mother. There was a conspicuous absence of bifidobacteria. In contrast, rectal cultures are normally sterile immediately after birth (*Rotimi* and *Duerden*, 1981; *Ekwempu* et al., 1982). Bacteria start to appear in faeces within 24 hours after birth. *Escherichia coli* and enterococci are frequently isolated even in the very first stool, especially if there has been a premature rupture of the foetal membranes. Colonisation by identical *E. coli* strains in mother and infant occurred in 18 out of 29 cases (*Gothefors* et al., 1976). Anaerobic bacteria belonging to the *Bacteroides* and *Bifidobacterium* genera can be detected in faeces within two days (*Mata* and *Urrutia*, 1971; *Patte* et al., 1979; *Rotimi* and *Duerden*, 1981; *Lejeune* et al., 1984). Bifidobacteria gradually appeared, and by the end of the first week, colonised all infants, and were completely dominating as long as breast-feeding continued in these studies. The initially high counts of *E. coli* declined during the first weeks of life. Starting at weaning, the microflora grows more complex and biochemically active (*Stark* and *Lee*, 1982; *Midtvedt* et al., 1988), but the 1000-fold dominance of anaerobic bacteria seen in adults, as well as adult diversity of bacterial species and biochemical functions, may not be attained for several years (*Ellis-Pregler* et al., 1975, *Norin* et al., 1985).

Conflicting results have been reached in some recent studies, where members of the *Bacteroides fragilis* group were the dominating anaerobic bacteria despite breast-feeding (*Simhon*

et al., 1982; *Lundequist* et al., 1985). In our studies (*Bennet* et al, 1986; *Bennet* and *Nord*, 1987), bifidobacteria still dominated, but not at all to the extent that was reported earlier.

Bacteroides species in neonatal faeces have been found to belong to the *B. fragilis* group, and in these studies *B. fragilis*, *Bacteroides distasonis*, *Bacteroides vulgatus* and *Bacteroides thetaiotaomicron* were the most common species (*Long* and *Swenson*, 1977; *Rotimi* and *Duerden*, 1981; *Bennet* and *Nord*, 1987). Among bifidobacteria, *Bifidobacterium adolescentis*, *bifidum*, *breve* (Sweden), *longum* (Japan), and *infantis* are the most common species (*Benno* et al., 1984; *Bennet* and *Nord*, 1987).

IMPACT OF FORMULA FEEDING ON INTESTINAL MICROFLORA

Type of feeding, i.e. breast milk versus formula, has for many decades been known to influence the faecal flora composition. In formula fed infants, *Bacteroides* dominated among the anaerobes and high counts of enterobacteria were found (*Haenel*, 1961; *Ellis-Pregler* et al., 1975; *Bullen* et al., 1976; *Bullen* et al., 1977, *Stark* and *Lee*, 1982; *Yoshioka* et al., 1983; *Benno*, 1984; *Lejeune* et al., 1984). *Rotimi* and *Duerden* (1981) found moderate numbers of bifidobacteria among infants fed breast milk supplemented with a milk preparation from a cow. *Sakata* et al. (1985) and *Kudinova* et al. (1982) reported that *Bifidobacterium* growth was proportional to the amount of breast milk given. *Lejeune* et al. (1984) found untreated breast milk to be superior to tyndallised and lyophilised breast milk in promoting *Bifidobacterium* growth. There are several factors in breast milk that may influence intestinal microflora. Among them IgA (produced by plasma cells "homing" in the breast glands after activation in the intestinal mucosa of the mother) (*Hanson* et al., 1984), viable white blood cells, lactoferrin, anti-inflammatory factors (*Goldman* et al., 1986), a low buffering capacity (facilitating the production of a low pH), and microorganisms.

Breast milk has been shown to protect against neonatal septicaemia (*Narayanan* et al., 1984). It does not prevent colonisation of the intestine by Gram-negative, potentially pathogenic bacteria, but breast milk IgA prevents contact between these microorganisms and the mucosal membranes (*Mata*, 1971; *Gothefors* et al., 1976; *Hanson* et al., 1984; *Stevenson* et al., 1985).

IMPACT OF CAESAREAN SECTION ON INTESTINAL MICROFLORA

Caesarean section leads to colonisation from the hospital environment rather than from the mother's vaginal and perineal flora. In an attempt to reveal the sources of colonisation of eight Caesarean section delivered newborn infants by *E. coli*, nearly 7000 cultures of samples from eight babies and their environment were analysed (*Lennox-King* et al., 1976). It was found that the most common sources were other infants via nurses' hands, but there was a surprisingly high degree of airborne contamination. Anaerobic colonisation, especially by *Bacteroides*, is delayed and if the infant is transferred to a neonatal unit there is an overgrowth of enterobacte-

ria other than *E. coli* (*Rotimi* et al., 1985; *Bennet* et al., 1986). In our studies (*Bennet*, 1987; *Bennet* and *Nord*, 1987), absence of *Bacteroides* persisted beyond two weeks of life, but both *Bifidobacterium* retrieval and *E. coli/Klebsiella* ratio were similar in vaginally and Caesarean section delivered infants.

In gastric aspirate obtained immediately after birth of babies delivered by Caesarean section after prolonged labour with rupture of membranes, there was no difference compared to vaginally delivered infants except that more streptococci were found (*Brook* et al., 1979).

IMPACT OF HOSPITALISATION AND PRE-TERM BIRTH ON INTESTINAL MICROFLORA

Hospitalisation, also without antibiotic treatment, produces changes of the normal microflora. Thus, colonisation by *Klebsiella*, *Proteus*, *Pseudomonas* and *Candida* was shown to occur in faeces of hospitalised adult patients after a few weeks (*LeFrock* et al., 1979a). Changes in intestinal flora as regards antimicrobial resistance of the bacteria, and also changes of bacterial species, have been shown to be followed by colonisation of both pharynx and skin by the same strains (*LeFrock* et al., 1979b, *Larson* et al., 1986). Also in new-born infants, intestinal colonisation by *Klebsiella*, as well as by other enterobacteria, occurs. It is much more pronounced after Caesarean section (*Long* and *Swenson*, 1977; *Bennet* and Nord, 1987).

In investigations of the anaerobic faecal microflora of hospitalised newborn infants, a delay in *Bifidobacterium* colonisation, a predominance of *Bacteroides*, especially after vaginal delivery, and sometimes an increased incidence of *Clostridium* species recovery is reported (*Graham* et al., 1976; *Goldmann* et al., 1978; *Blakey* et al., 1982; *Rotimi* and *Duerden*, 1982; *Stark* and *Lee*, 1982, *Sakata* et al., 1985). In some of these studies, however, breast milk was not used, or heated to l00°C, or antibiotic treatment given. We found no differences in the anaerobic microflora between term and pre-term infants that could not be explained by neither antibiotic treatment nor a higher rate of Caesarean section in the latter group (*Bennet* and *Nord*, 1987). In the study by *Sakata* et al. (1985), similar results were obtained except for a delay of detection of anaerobes in very low birth weight infants. This was supposed to be a result of the very small amounts of breast milk tolerated by such infants during the first weeks of life. In conclusion, the control of the microflora seems to be intact also in very immature infants, but is easily disturbed by iatrogenic factors.

IMPACT OF ANTIMICROBIAL TREATMENT ON INTESTINAL MICROFLORA

Current knowledge of the effects of various antibiotics on the intestinal flora in adults has been summarised by *Nord* and co-workers (1986). Such effects are the net result of the antimicrobial spectrum of the drug, concentrations in bile, saliva and other secretions, re-absorption, faecal binding,

and antimicrobial inactivation. The effects usually measured are:

1. suppression of anaerobic bacteria,
2. new colonisation, and
3. tendency to induce resistance in bacteria.

Clindamycin, erythromycin and also ampicillin have a strong influence on the intestinal flora, whereas narrow-spectrum penicillins such as phenoxymethylpenicillin and benzylpenicillin have minor effects in clinical doses. Some modern cephalosporins are excreted to a large extent in bile and produce profound changes of the flora (*Bodey* et al., 1983). Aminoglycosides, on the other hand, are excreted in the urine and have no effect on intestinal microflora when given parenterally.

We have studied the influence of various common antibiotic regimens on both aerobic and anaerobic intestinal flora of new-born infants (*Bennet*, 1987). During treatment, there was a suppression of susceptible aerobic bacteria in a predictable way according to the antibacterial spectrum of the drug used. When cephalosporins were used, an overgrowth of enterococci occurred. There was a colonisation by and overgrowth of *Klebsiella* in all treatment groups, including those treated with the narrow-spectrum benzylpenicillin, cloxacillin and flucloxacillin. Other investigators have also found frequent colonisation with various aerobic Gram-negative rods, e.g., *Citrobacter*, *Pseudomonas* and *Proteus* during antibiotic therapy (*Graham* et al., 1976; *Goldmann* et al., 1978; *Lambert-Zechovsky* et al., 1984).

In our study, all regimens led to undetectable levels of anaerobic bacteria in 80-90% of the patients. In half of the remaining children, *Clostridium* species was the only anaerobic microorganism - a condition that was never found in untreated infants.

After antibiotic treatment, there was a slow but steady normalisation of the intestinal flora (*Bennet* et al., 1986). There was a regrowth of bifidobacteria but a continuing absence of *Bacteroides*, also in vaginally delivered infants. The *E. coli/Klebsiella* ratio slowly reverted back to one of *E. coli* dominance (*Bennet* and *Nord*, 1987). There were, however, a few cases where anaerobic bacteria remained absent for several weeks, and in these infants a heavy growth of *Klebsiella* continued. Among the treated infants, there were no differences relating to mode of delivery.

The fact that *Bacteroides* species do not re-establish after antibiotic treatment suggests that they are truly eradicated from the intestinal tract and that little transmission of *Bacteroides* occurs from the external environment. Clinically, *Bacteroides* infections are very rare in new-borns. In only one of 329 cases of neonatal septicaemia in our neonatal intensive care unit during 1979-1983, *Bacteroides* was isolated from blood (*Bennet* et al., 1985). Since *Bacteroides* has usually been shown to be rare in the faeces of new-born infants, its failure to become re-established may not be of any disadvantage.

NEONATAL SEPTICAEMIA

The increasing survival of high-risk infants has created a new population of patients, extremely vulnerable to infection. There are several reasons for this increased infectious risk, both in the environment and within the infants themselves. The abnormal colonisation of various anatomical sites of NICU patients is similar to what is known from adults (*Goldmann*, 1981; *Morgan*

et al., 1984; *Chugh* et al., 1985). The role of hands as carriers and even reservoirs of Gram-negative bacteria has been pointed out by *Knittle* et al. (1975).

Neonatal septicaemia has remained a serious problem. It has become evident that the clinical picture of this disease is changing, both as regards bacterial aetiology and patient characteristics (Davies and *Gothefors*, 1984; *Bennet* et al., 1985; *Bennet* et al., 1987a,b). Many cases of septicaemia are nowadays caused by staphylococci and group B streptococci, probably emanating from the skin and the mother's cervical flora. However, Gram-negative infections remain a serious problem and are coupled to high mortality and rate of sequelae in survivors (*Bennet* et al., 1989). It is likely that Gram-negative infections often start with antibiotic-induced intestinal overgrowth as demonstrated by *Mathieu* et al. (1984). In our intensive care unit, *Klebsiella* infections were always preceded by antibiotic treatment or Caesarean section (*Bennet* et al., 1987a).

IMPLANTATION OF MICROORGANISMS IN INTESTINAL MICROFLORA

Our ignorance of detailed rules of intestinal microbial ecology is reflected when it comes to methods used to "conventionalise" germfree animals. Complete normalisation of the physiological peculiarities of these animals has so far been achieved only by administration of faeces or intestinal contents from conventional animals without intervening cultures.

There are several animal studies showing an ability of the normal microflora to prevent colonisation by other microorganisms. Here, too, undefined caecal contents have been most successful (*Rantala* and *Nurmi*, 1973; *Berg*, 1980a,b; *Dubos* et al., 1984, *Soerjadi-Liem* et al., 1983). Specific mixtures of 48 and 239 strains, respectively, have been shown to replicate this effect (*Schneitz* et al., 1981; *Impey* et al., 1982).

Starting with fermented milk products during the first half of this century, bacterial interference programs or "bacteriotherapy" have also been attempted in humans, in order to treat more or less well-defined gastrointestinal disorders. It seems unlikely that success in humans should be obtained with very simple cultures of only one or a few anaerobic species. Recently, *Reuman* et al. (1986) reported no effect of a *Lactobacillus acidophilus* preparation on colonisation of low-birthweight infants by resistant Gram-negative bacteria. Yet, there are publications that report good results from oral therapy with lactobacilli (*Prado* et al., 1980; *Zoppi* et al., 1982).

Another approach is to give one apathogenic bacterial species, which is ecologically similar to the offending one, as demonstrated with nasopharyngeal alpha-streptococci by *Sprunt* et al. (1980). *Duval-Iflah* et al. (1982) reported the creation of a barrier function against colonisation by resistant *E. coli* by giving another strain of this species to new-born infants.

LITERATURE

Bennet, R., Eriksson, M., Melen, B., and Zetterström, R.: Changes in the incidence and spectrum of neonatal septicemia during a fifteen-year-period. Acta Paediatr. Scand. 74, 687-690 (1985).

Bennet, R., Eriksson, M., Nord, C.E., and Zetterström, R.: Fecal bacterial microflora of newborn infants during intensive care management and treatment with five antibiotic regimens. Pediatr. Infect. Dis. 5, 533-539 (1986).

Bennet, R., Eriksson, M., and Zetterström, R.: Bacterial etiology of neonatal septicemia in relation to prior antibiotic treatment. Acta Paediatr. Scand. 76, 673-674 (1987a).

Bennet, R., Eriksson, M., and Zetterström, R.: Neonatal septicemia: Comparison of onset and risk factors during three consecutive 5-year periods. Acta Paediatr. Scand. 76, 361-362 (1987b).

Bennet, R., and Nord, C.E.: Development of the fecal anaerobic microflora after Caesarean section and treatment with antibiotics in newborn infants. Infection 15, 332-336 (1987).

Bennet, R., Bergdahl, S., Eriksson, M., and Zetterström, R.: The outcome of neonatal septicemia during fifteen years. Acta Paediatr. Scand. 78, 40-43 (1989).

Benno, Y., Sawada, K., and Mitsuoka, T.: The intestinal microflora of infants: Composition of fecal flora in breastfed and bottle-fed infants. Microbiol. Immunol. 28, 975-986 (1984).

Berg, R.: Inhibition of *Escherichia coli* translocation from the gastrointestinal tract by normal cecal flra in gnotobiotic or antibiotic-decontaminated mice. Infect. Immun. 29, 1073-1081 (1980a)

Berg, R.: Mechanisms confining indigenous bacteria to the gastrointestinal tract. Am. J. Clin. Nutr. 33, 2472-2484 (1980b).

Blakey, J.L., Lubitz, L., Barnes, G.L., Bishop, R.F., Campbell, N.T., and Gillam, G.L.: Development of gut colonization in preterm neonates. J. Med. Microbiol. 15, 519-529 (1982).

Brook, I., Barett, C., Brinkman, C., Martin, W., and Finegold, S.: Aerobic and anaerobic bacterial flora of the maternal cervix and newborn gastric fluid and conjunctiva: A prospective study. Pediatrics 63, 451-455 (1979).

Bodey, G., Fainstein, V. Garcia, I., Rosenbaum, B., and Wong, Y.: Effect of broad-spectrum cephalosporins on the microbial flora of recipients. J. Infect. Dis. 148, 892-897 (1983).

Bullen, C.L., Tearle, P.V., and Stewart, M.G.: The effect of "humanised" milks and supplemented breast feeding on the fecal flora of infants. J. Med. Microbiol. 10, 403-413 (1977).

Bullen, C.L., Tearle, P.V., and Willis, A.T.: Bifidobacteria in the intestinal tract of infants: An *in-vivo* study. J. Med. Microbiol. 9, 325-333 (1976).

Chught, T.D., Ghaffoor, M.B., Kuruvilla, A.C., and Bishibishi, E.A.: Colonization and infections of neonates by *Klebsiella pneumoniae* in an intensive care unit. J. Trop. Pediatr. 31, 200-203 (1985).

Davies, P., and Gothefors, L.: bacterial infections in the fetus and newborn infant. W.B. Saunders, Philadelphia, p. 3 (1984).

Dubos, F., Martinet, L., Dabart, J., and Ducluzeau, R., Immediate post-natal inoculation of a microbial barrier to prevent neonatal diarrhea induced by *Clostridium difficile* in young conventional and gnotobiotic hares. Am. J. Vet. Res. 45, 1242-1243 (1984).

Duval-Ifflah, Y., Ouriet, M.-F., Moreau, C., Daniel, N., Gabilan, J.-C., and Raibaud, P.: Implantation précoce d'une souche de *Escherichia coli* dans l'intestin du nouveau-né humain: Effet de Barrière vis-à-vis de souche de *E. coli* antibioresistants. Ann. Microbiol. (Inst. Pasteur) 133A, 393-408 (1982).

Ekwempu, C., Lawande, R., and Egler, L.: bacterial colonisations of varous sites at birth of babies born in Zaira. J. Infect. 5, 177-181 (1982).

Ellis-Pregler, R.B., Crabtree, C., and lambert, H.P.: The faecal flora of children in the United Kingdom. J. Hyg. Camb. 75, 135-142 (1975).

Goldmann, D., Leclair, J., and Macone, A.: Bacterial colonization of neonates admitted to an intensive care environment. J. Pediatr. 93, 288-293 (1978).

Goldmann, D.: Bacterial colonization and infection in the neonate. Am. J. Med. 70, 417-422 (1981).

Goldman, A., Thorpe, L., Goldblum, R., and Hanson, L.: Anti-inflammatory properties of human milk. Acta Paediatr. Scand. 75, 689-695 (1986)

Gothefors, L., Carlsson, B., Ahlstedt, S., Hanson, L.Å., and Winberg, J.: Influence of maternal gut flora and colostral and cord serum antibodies on presence of *Escherichia coli* in faeces of the newborn infant. Acta Paediatr. Scand. 65, 225-232 (1976).

Graham, J., Taylor, J., and Davies, P.A.: Some aspects of bacterial colonisation in ill, low-birth, and normal newborns. In: Intensive care in the newborn (Eds.: Stern, L., Friis-hansen, B., and Kildebeerg, P.). Masson, New York, 59-72 (1976).

Haenel, H.: Some rules in the ecology of the intestinal microflora of man. J. Appl. Bact. 24, 242-252 (1961).

Hanson, L.A., Ahlstedt, S., Andersson, B., Cruz, J.R., Dahlgren, U., Fallstrom, S.P., Porras, O., Svanborg Eden, C., Soderstrom, T., and Wettergren, B.: The immune response of the mammary gland and its significance for the neonate. Ann. Allergy 53, 576-582 (1984).

Knittle, M., Eitzman, D., and Bayeer, H.: Role of hand contamination of personel in the epidemiology of gramnegative nosocomial infections. J. Pediatr. 86, 433-437 (1975).

Kudinova, T., Elizarova, I., Razumovskaja, I., and Kozlova, E.: Early onset of breast-feeding: The formation of intestinal microflora in normal newborns within the first week of life. Akush. Ginekol. 9, 22-25 (1982).

Lambert-Zechovsky, N., Bingen, E., Bourillon, A., Aujard, Y., and Mathieu, H.: Effects of antibiotics on the microbial intestinal ecosystem. Dev. Pharmacol. Ther. 7 (suppl. 1), 150-157 (1984).

Larson, E., McGinley, K., Foglia, A., talbot, G., and Leyden, J.: Composition and antimicrobic resistance of skin flora in hospitalized and healthy adults. J. Clin. Microbiol. 23, 604-608 (1986).

LeFrock, J., Ellis, C., and Weinstein, L.: The impact of hospitalization on the aerobic fecal microflora. Am. J. Med. Sci. 277, 269-274 (1979a).

LeFrock, J., Ellis, C., and Weinstein, L.: The relation between aerobic fecal and oropharyngeal microflora in hospitalized patients. Am. J. Med. Sci. 277, 275-280 (1979b).

Lejeune, C., Bourillon, A., Boussogant, Y., and De Papillerets, F.: Sequential development of the intestinal flora in newborn infants: A quantitative differential analysis. Dev. Pharmacol. Ther. 7 (suppl. 1), 138-143 (1984).

Lennox-King, S., O'Farrol, S., bettelheim, K., and Shooter, R.: *Escherichia coli* isolated from babies delivered by caesarean section and their environment. Infection 4, 439-445 (1976).

Long, S.S., and Swenson, R.M.: Development of anaerobic fecal flora in healthy newborn infants. J. Pediatr. 91, 298-301 (1977).

Lundequist, B., Nord, C.E., and Winberg, J.: The composition of the fecal microflora in breastfed and bottle fed infants from birth to eight weeks. Acta Paediatr. Scand. 74, 45-51 (1985).

Mata, L., and Urrutia, J.: Intestinal colonization of breastfed children in a rural area of low socioeconomic level. Ann. NY Acad. Sci. 93-109 (1971).

Morgan, M.E.I., Hart, C.A., and Cooke, R.W.I.: *Klebsiella* infection in a neonatal intensive care unit: Role of bacteriological surveillance. J. Hosp. Infect. 5, 377-385 (1984).

Narayanan, I., Prakash, K., Murthy, N.S., and

Guiral, V.V.: Randomised control trial of effect of raw and holder pasteurised human milk and of formula supplements on incidence of neonatal infection. Lancet ii, 1111-1113 (1984).

Nord, C.E., Heimdahl, A., and Kager, L.: Antimicrobial induced alterations of the human oropharyngeal and intestinal microflora. Scand. J. Infect. Dis. 46, 64-72 (1986).

Norin, K.E., Gustafsson, B.E., Lindblad, B.S., and Midtvedt, T.: The establishment of some microflora associated biochemical characteristics in feces from children during the first years of life. Acta Paediatr. Scand. 74, 207-212 (1985).

Patte, C., Tancrède, C., Raibaud, P., and Ducluzeau, R.: Premières étapes de la colonisation bacteriènne du tube digestiv du noveau-né. Ann Microbiol. (Pasteur) 130A, 69-84 (1979).

Prado, V., Aguero, M.E., Ernst, Y., Marin, P., and Diaz, M.C.: Efectos de la administracion de lactobacilos sobre la flora intestinal en lactantes tratados con antibioticols de amplio espectro. Rev. Chil. Pediatr. 51, 9-12 (1980).

Rantala, M. and Nurmi, E.: Preventin of the growth of *Salmonella infantis* in chicks by the flora of the alimentary tract of chickens. British Poultry Science 14, 627-630 (1973).

Reuman, P., Duckworth, D., Smith, K., Kagan, R., Bucciarelli, R., and Ayoub, E.: Lack of effect of *Lactobacillus* on gastrointestinal bacterial colonization in premature infants. Pediatr. Infect. Dis. 5, 663-668 (1986).

Rotimi, V.O. and Duerden, B.I.: The bacterial flora of neonates with congenital abnormalities of the gastro-intestinal tract. J. Hyg. (Lond.) 88, 69-81 (1982).

Rotimi, V.O. and Duerden, B.I.: The development of the bacterial flora in normal neonates. J. Med. Microbiol. 14, 51-62 (1981).

Rotimi, V.O., Olowe, S.A., and Ahmed, I.: The development of bacterial flora of premature neonates. J. Hyg. (Lond.) 94, 309-318 (1985).

Sakata, H., Yoshioka, H., and Fujita, K.: Development of the intestinal flora in very low birth weight infants compared to normal full-term newborns. Eur. J. Pediatr. 144, 186-190 (1985).

Schneitz, C., Seuna, E., and Rizzo, A.: The anaerobically cultured cecal flora of adult fowls that protects chickens from *Salmonella* infections. Acta Pathol. Microbiol. Scand. Sect. B 89, 109-116 (1981).

Simhon, A., Douglas, J.R., Drasar, B.S., and Soothill, J.F.: Effect of feeding infants' faecal flora. Arch. Dis. Child. 57, 54-58 (1982).

Soerjadi-Liem, A., Snoeyenbos, G., and Weinack, O.: Comparative studies on competitive exclusion of three isolates *of Campylobacter fetus* subsp. *jejuni* in chickens by native gult microflora. Avian Diseases 28, 139-146 (1983).

Sprunt, K., Leidy, G., and Redman, W.: Abnormal colonization of neonates in an ICU: Conversion to normal colonization by pharyngeal implantation of alpha-hemolytic streptococcus strain 215. Pediatr. Res. 14, 308 (1980).

Stark, P.L. and Lee, A.: The bacterial colonization of the large bowel of pre-term low birth weight neonates. J. Hyg. Camb. 89, 59-67 (1982).

Stark, P.L. and Lee, A.. The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. J. Med. Microbiol. 15, 189-203 (1982).

Stevenson, D., Young, C., Kerner, J., and Yeager, A.: Intestinal flora in the second week of life in hospitalized preterm infants fed stored-frozen breast-milk or a proprietary formula. Clin. Pediatr. 24, 338-341 (1985).

Yoshioka, H., Iseki, K., and Fujita, K.: Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. Pediatrics 72, 317-321 (1983).

Zoppi, G., Deganello, A., Benoni, G., and Saccomani, F.: Oral bacteriotherapy in clinical practice I. The use of different preparations in infants treated with antibiotics. Eur. J.Pediatr. , 18-21 (1982).

Old Herborn University Seminar Monograph 4:
Host-microflora interactions in the first years after birth.
Editors: Peter J. Heidt, Rial D. Rolfe, Volker Rusch, and Dirk van der Waaij.
Institute for Microbiology and Biochemistry, Herborn-Dill, Germany: 22-32 (2002).

BOVINE LACTOGENIC IMMUNITY: A CONCEPT WHOSE TIME HAS COME (?)

MARY BOESMAN-FINKELSTEIN, and RICHARD A. FINKELSTEIN

Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, MO 65212, USA

SUMMARY

Since the pioneering studies of Paul Ehrlich, it has been widely accepted that breast-fed infants fare better than their non-breast-fed counterparts with regard to their resistance to infectious diseases. Indeed breast milk has many antimicrobial components, including antibodies, which could serve to protect the infant. Studies are reviewed which indicate that although the antimicrobial spectrum of human milk *in vitro* is quite diverse, not all pathogenic species are susceptible, susceptibility varies among strains of a species, and the potency of the antimicrobial activity varies from mother to mother. In addition, despite intensive efforts by national and international organisations, a significant proportion of infants are not breast-fed. This is of particular significance in lesser-developed countries where diarrhoeal disease is rampant. Studies with laboratory animal models, and a few studies with adult volunteers or in hospital nurseries, have shown that orally administered antibody of human or bovine origin can be markedly protective, if not therapeutic, against diarrhoeal disease caused by rotavirus and by enterotoxigenic *Escherichia coli*, and against necrotising enterocolitis in premature infants. Orally administered purified bovine colostral immunoglobulin, from newly parturient cows immunised with cholera enterotoxin, the cholera toxin related enterotoxin from *E. coli*, or outer membrane proteins of *Vibrio cholerae* protected infant rabbits against lethal direct intra-intestinal challenge with virulent *V. cholerae*. These and other experimental observations, published studies, and current analysis suggest that the concept of passive bovine lactogenic immunity, i.e., the oral administration of purified colostral or milk immunoglobulin from hyperimmunised cows, merits further controlled evaluation in field studies and could offer a means of protecting infants who are not breast-fed and of complementing and supplementing the immunity of infants.

Almost a century ago, *Paul Ehrlich* (1892) established unequivocally, in a series of brilliant experiments involving four or so mice apiece, that immunity - to abrin and ricin - was transmitted not by inheritance from immunised father mice but from immunised mother mice (Table 1). The latter immunity wanes with the age of the offspring. Furthermore, infant mice born of non-immune mice and nursed from mothers immunised against abrin or ricin were protected against toxin challenge, whereas infants born of immune mother mice and nursed by non-immune mice showed no protection

Table 1: Protection is transmitted by immune mothers but not by immune fathers[1]

Father: Mother:	Immune Normal	Normal Immune	Age at challenge (days)	Antigen (imm/chall)	Challenge (x lethal dose)
	5/6[2]	0/3	21-45	abrin/abrin	0.2-1.33
		0/3	21-45	ricin/ricin	4-10
		9/11	56-113[3]	abrin/abrin	0.25-4.00
		3/4	86-108[3]	ricin/ricin	1-2

[1]Data modified from *Ehrlich*, 1982
[2]Dead/Total
[3]After nursing period

(Table 2). "Wissen Sie ..... verstehen Sie," as Ehrlich would have said (*Marquardt*, 1951). [Coincidentally, Ehrlich studied abrin and ricin because he felt that these recently discovered toxic lectins were related to the bacterial toxins, diphtheria and tetanus toxins, which had also been recently described. *Ehrlich* (1891) was the first to show that abrin and ricin were immunologically different proteins].

Since their advent, the survival of mammals has depended upon the passive transfer of immunity from mother to offspring, whether transplacentally prior to birth, postnatally via breast milk, or both. This held true for humans as well until the domestication of animals made their milk available as a substitute source of nutrition. As early as 1900 B.C., Hammurabi's code regulated the practice of paid "wet nursing"; i.e., the nursing of another person's infant. Two centuries B.C., there began to appear evidence of feeding cups in graves of infants throughout Europe (*Lawrence*, 1989) and feeding horns (cow) from the twelfth century were found in the basement of St. Bartholomew's Hospital in London (*Walker-Smith*, 1975). In ancient Sparta, the wife of the king was obliged by law to nurse her oldest son. If he was nursed by a stranger, he lost his line of succession to the monarchy. It is said that Hippocrates wrote, "One's own milk is beneficial, other's harmful" (*Lawrence*, 1989).

During the Middle Ages, well-to-do English mothers did not nurse their infants. Although this was already recognised as a means of birth control, they preferred to have as many as 12-20 children rather than "spoil their figures and make them old before their time" (*Fildes*, 1986). In Eighteenth-Century-France around the time of the Revolution, breast-feeding was not customary and children were either given to wet nurses or fed artificially (*Lawrence*, 1989). Until the last several decades, women were urged to raise their children "scientifically" with a diet comprised of cod liver oil, orange juice, and artificial feeding (*Apple*, 1987; *Lawrence*, 1989).

Following the observations of Ehrlich on the importance in mice of the passive immunity provided by milk and with the emergence of the field of immunology, comparisons began to be made (as early as 1895 in Berlin) on the mortality rate differences between breast-fed and artificially fed infants (*Knodel*, 1977). The campaign to promote breast-feeding began. Since then, it has become increasingly evident in many studies world-wide (*Jelliffe* and *Jelliffe*, 1988) that breast-feeding the infant for at least 6 months (preferably

Table 2: Immunity is transmitted to infant mice from normal mothers by nursing immune mothers[1]

Foster mothers		Challenge	Challenge
Immune	Normal	antigen	(x lethal dose)
1/5[2]		abrin	1.25-40
0/6		ricin	2.25-40
	6/6	abrin	1.25-40

[1]Data modified from *Ehrlich*, 1892
[2]Dead//Total

for 1 year) until his immune system becomes fully operational is perhaps the one of the most important things a mother can give her child. This passive immunity, in the form of immunoglobulins, immuno-important cell populations, and non-specific antimicrobial agents, along with the nearly perfectly evolved nutrition, affords an infant a relatively protected state in which to grow relatively unimpeded by constant bouts with severe life-threatening diseases, particularly diarrhoeal diseases. In addition to the antimicrobial substances found in breast milk, the exposure of the infant to entero- and other pathogens, in diet and in environment, is reduced.

Although the vast majority of studies which have demonstrated that breast-feeding reduces infant morbidity and mortality have been flawed in one way or another - because of understandable lack of appropriate controls or other variables - the volume of the evidence in favour of breast-feeding is convincing (*Feachem* and *Koblinsky*, 1984; *Jason* et al., 1984; *Kovar* et al., 1984; *Mata*, 1978, 1986). Thus, it has become universally accepted that breast-fed babies fare better than formula-fed babies with regard to resistance to infectious diseases and especially to diarrhoeal diseases. Each year, diarrhoeal diseases affect over 150 million and kill more than 4 million children under the age of 5 in the lesser-developed countries of the world (*Cleason* and *Merson*, 1990; *Snyder* and *Merson*, 1982).

If we accept that breast-feeding is indeed beneficial in terms of protection against infectious diseases, in addition to the reduction of exposure to pathogens in the environment - in contaminated food and water - what are the protective mechanisms of breast-feeding? Table 3 lists many of an ever-increasing number of antimicrobial components, which have been observed in human milk. Although many have been shown to be active in *in vitro* tests, their potential clinical importance remains to be evaluated in experimental animal models or in human beings. Of the components listed, the immunoglobulins are the most likely to be of practical significance. They have been demonstrated to neutralise bacterial toxins, to inactivate viruses, to prevent bacterial adherence to host cells, and, in some instances, to have direct antibacterial effects - sometimes in combination with other factors such as lactoferrin, lysozyme, and perhaps complement components of the alternative pathway.

As summarised in Table 4, which includes studies from our own (*Boesman-Finkelstein* and *Finkelstein*, 1985; *Dolan* et al., 1986, 1989) as well as other laboratories, mothers' milk has a broad spectrum of antimicrobial activity which ranges from the upper

Table 3: Antimicrobial components of human milk*

Immunoglobulins
1. SIgA
2. Other Ig Classes
Bifidobacterium bifidus growth factor
Lactoferrin
Lysozyme
Lactoperoxidase
Alpha-2 macroglobulin
Alpha-1 antitrypsin
Ribonuclease
Lipid
1. Free unsaturated fatty acids and monoglycerides
2. Gangliosides (GM1)
3. Glycolipid receptor analogues
Carbohydrate
1. Oligosaccharide receptor analogues
2. Non-lactose carbohydrates
Cells
1. T and B lymphocytes
2. Neutrophils
3. Macrophages

*Table derived from *Goldman* et al. (1985); *Hanson* et al. (1988); *Lawrence* (1989); and *May* (1988).

respiratory through the enteric microflora. In our laboratory, the following potential pathogens have been shown to be inhibited by pooled human whey: Groups B and D streptococci (markedly bactericidal); coagulase-positive and coagulase-negative staphylococci (bacteriostatic); *Hemophilus influenzae* (markedly bactericidal); *E. coli* (enteric isolates were markedly inhibited, whereas systemic isolates were not); *Shigellae* (8/10 strains inhibited); *Salmonella typhimurium* (all inhibited by whey with variation in degree from strain to strain); blood isolates of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens* (inhibition varied from strain to strain); and *Vibrio cholerae* (marked inhibition). *Campylobacter* strains, in our study were not markedly inhibited in whey under the micro-aerophilic culture conditions used but, interestingly, they were killed in commercial infant formula used as a control. On the other hand, *Ruiz-Palacios* et al. (1990) have just demonstrated an association between *Campylobacter* antibodies in human milk and prevention of diarrhoea caused by *Campylobacter*. This apparent contradiction indicates that caution should be exercised when extrapolating from results obtained *in vitro*.

It should be noted, however, that not all strains of a given species of pathogenic bacteria are equally susceptible to the antimicrobial activity of, in this case, pooled whey from Missouri mothers (*Dolan* et al., 1986, 1989). Furthermore, the potency of the antimicrobial effects of whey varies from individual to individual - probably in large measure dependent on the prior immunologic experience of each mother (*Boesman-Finkelstein* and *Finkelstein*, 1985).

Thus, acknowledging that the health of breast-fed babies will generally be better than those who are not, one might predict that some individual mothers' breast-feeding might not be as

Table 4: Microorganisms inhibited by human breast milk *in vitro* or by breast feeding

Bacteria	
Campylobacter	Klebsiellae
Chlamydia	Pasteurellae
Clostridium botulinum	Pneumococci
Clostridium difficile	Pseudomonas
Corynebacterium diphtheriae	Salmonellae
Escherichia coli	Serratia
enterotoxigenic	Shigellae
enteropathogenic	Staphylococci
enterohemoragic	Streptococci
enteroadherent	Vibrio cholerae
Hemophilus	
Viruses	
Coxsackie	Polio
Cytomegalovirus	Parainfluenza
Dengue	Respiratory syncytial
Herpes simplex	Rotavirus
Influenza	Rubella
Japanese B encephalitis	Semliki forest
Mumps	
Parasites	
Ascaris lumbricoides	Schistosoma mansoni
Cryptosporidium	Trichomonas vaginalis
Entamoeba histolytica	Trypanosoma rhodesiense
Giardia lamblia	
Fungi	
Candida albicans	

protective as breast-feeding by other individual mothers and that some pathogens or strains of pathogens might be more or less responsive than others.

In addition, despite the weight of evidence and the global emphasis by UNICEF and the World Health Organisation on the importance of breast-feeding, the vast majority of mothers, particularly in the lesser-developed countries of the Third World, are not breast-feeding and, if they are, not long enough. Indeed, UNICEF has recently acknowledged that its efforts to promote breast-feeding have been less than successful:

> "Breast-feeding appears to be on the decline in many developing nations as commercial pressures, the use of milk powder and feeding bottles in hospitals...., and the increased participation of women in the labour force, all conspire to make bottle feeding seem the attractive option. The continuation of this trend would be disastrous (*Grant*, 1990)."
>
> If the trend continues, what then can be done to protect the infants who are not breast-fed (and to complement and supplement the immunity of those who are)?

Among the antimicrobial components in milk listed previously (Table 3), the antibody-containing immunoglobulins head the list of potentially useful agents. *Peterson* and *Campbell* (1955) first conceived of the use of passive oral immunotherapy. Subsequently, *Hanson* (1961) described a secretion-

unique immunoglobulin, now known as secretory immunoglobulin A (SIgA) as a major protein in human milk. Beginning in 1958, a number of studies have been performed to evaluate the effects of orally administered antibodies, in one form or another. These attempts may be divided into those directed toward therapy of existing disease and those directed to prophylaxis against disease in the present or future.

Svirsky-Gross (1958) and *Tassovatz* and *Kotsitch* (1961) successfully showed that passive oral administration of human milk successfully stopped epidemics of *Escherichia coli* O111:B4 in their new-born nurseries. Recognising that the immunoglobulin concentration in colostrum is higher than that in milk, *Larguia* et al. (1977) controlled an outbreak of enteropathogenic *E. coli* diarrhoea in a premature nursery by oral administration of 5 ml/kg/day of a pool of colostrum from several mothers. *Narayanan* et al. (1980) showed significant protection against infection in 32 high-risk low-birth-weight infants by partially feeding human breast-milk (breast-milk during the day, formula at night) compared with 38 infants receiving formula alone. *Barnes* et al. (1982) evaluated the protective effect of orally administered commercial pooled human serum gammaglobulin in a group of 75 low-birth-weight infants in a nursery where rotavirus was known to be endemic. In the placebo group, 6 of 11 babies developed severe rotavirus diarrhoea whereas only 1 of 14 given Ig was affected. In a recent study by *Eibl* et al. (1988), the development of necrotising enterocolitis in low-birth-weight new-borns was prevented by oral administration pooled human serum immunoglobulins (Cohn Fraction II containing 75% IgG and 25% IgA). There were no cases in the 88 infants receiving Ig compared with 6 in the control group of 91. In that study, oral Ig administration was completely protective.

The newly parturient cow may be regarded as an immunoglobulin-producing factory that secretes into its colostrum kilogram amounts of IgG1, the bovine milk immunoglobulin counterpart of the human secretory IgA (*Lascelles* and *McDowell*, 1974). As with human SIgA (*Kenny* et al., 1967; *Lindh*, 1975), bovine IgG1 has been shown to be relatively protease-resistant and immunologic reactivity is retained after passage through the intestinal tract (*Hilpert* et al., 1974, 1975; *McClead* and *Gregory*, 1984). A number of studies have examined the therapeutic/protective effects of feeding hyperimmune bovine immunoglobulin. In this laboratory (*Boesman-Finkelstein* et al., 1989), hyperimmune bovine IgG1 was purified from the colostrum of newly parturient cows who had been immunised with cholera toxin (CT), *E. coli* heat-labile toxin (LT) and *V. cholerae* outer membrane proteins. Administered by gastric feeding tube in infant feeding formula, all were shown to protect 6-day-old infant rabbits from diarrhoea following intra-intestinal challenge with virulent cholera vibrios. Both protection studies, as well as immunologic analysis by checkerboard immunoblotting (*Kazemi* and *Finkelstein*, 1990), a technique recently developed in our laboratory, indicated that homologous preparations are more reactive (unpublished results).

Mietens et al. (1979) evaluated the therapeutic effect of orally administered hyperimmune bovine milk immunoglobulin concentrate (MIC, containing about 40% Ig), containing antibodies to 14 serologically different strains of enteropathogenic *E. coli*, on 60 infants (ages 10 days to 18 months) suffering from *E. coli* diarrhoea. No therapeutic effect was demonstrated although the period of excretion of the

homologous serotypes of *E. coli* was reduced. A similar attempt to treat infants with rotavirus diarrhoea with an MIC containing antibodies to 4 different human rotavirus serotypes was also unsuccessful although the higher titred anti-rotavirus preparation did reduce the excretion of virus (*Brussow* et al., 1987; *Hilpert* et al., 1987). Recently, *McClead* et al. (1988) gave purified bovine immunoglobulin containing anti-cholera toxin antibodies to patients with active cholera. Although toxin neutralising activity was found in the stools of most of the patients, orally administered antibody did not alter the course of active cholera diarrhoea.

Although feeding antibody-containing Ig preparations has quite clearly not been successful therapeutically, administration of such Ig prophylactically would seem to be more logical since the disease may not be reversible by antibody whereas the prophylactic effect of feeding human milk against diarrhoeal disease in infants has long been known. Recently, *Tacket* et al. (1988) demonstrated that a MIC containing antibodies against CT, LT, and a variety of enteropathogenic strains of *E. coli* was protective in studies using adult American volunteers who were challenged with enterotoxigenic *E. coli*. None of the 10 volunteers receiving the immune colostrum had diarrhoea when challenged with 10^9 colony forming units of enterotoxigenic *E. coli* H10407, whereas, 9 of the 10 receiving control colostrum did. All excreted the challenge *E. coli* strain. *Ebina* et al. (1985) orally administered 20 ml of hyperimmune bovine anti-rotavirus colostrum daily to 6 infants in an orphanage while 7 control infants received 20 ml of commercial milk. After a period of 1 month, 6 of the 7 control infants had developed rotavirus diarrhoea, whereas 5 of the 6 colostrum-fed infants were free of diarrhoea. Two of these 5, interestingly, developed demonstrable complement-fixing antibodies to rotavirus during this period. In another group in the same study, hyperimmune colostrum administration had no therapeutic effect on rotavirus infection. *Davidson* et al. (1989) showed that infants fed bovine colostrum from cows immunised with 4 serotypes of human rotavirus were protected against nosocomial rotavirus infection. In that study, 9 of 65 control children, but none of the 55 colostrum-fed infants developed rotavirus diarrhoea. Although it was not altogether clear that antibody, rather than other components of the colostrum, was responsible (*Boesman-Finkelstein* and *Finkelstein*, 1989), it was clear that the colostrum preparation protected against rotavirus diarrhoea.

The studies described above have shown that either bovine or human antibodies are efficacious prophylactically, but they were generally not effective therapeutically. Immunologically compromised subjects, however, may be an exception to this rule. Hyperimmune bovine colostrum from cows immunised with *Cryptosporidium* oocysts administered via a naso-gastric tube was reported to be effective in the treatment of 4 immunodeficient patients – two of whom had AIDS - with chronic diarrhoea caused by *Cryptosporidium*: 1) a 3-year-old hypogammaglobulinaemic was treated with 200 ml hyperimmune colostrum/day for 12 days (*Tzipori* et al. 1986, 1987); 2) a 38-year-old AIDS patient received 500 ml/day colostrum for 21 days (*Tzipori* et al., 1987); and 3) a 4-year-old on immunosuppressive therapy for acute lymphoblastic leukaemia received 500 ml colostrum/day for 10 days (*Tzipori* et al., 1987). They recovered from their diarrhoea within 3-5 days. Another recent report (*Ungar* et al., 1990) also showed therapeutic/prophylactic effi-

cacy of using hyperimmune bovine anti-*Cryptosporidium* oocysts in the treatment of AIDS patients. Colostrum was administered continuously (20 ml/hour) via a naso-duodenal tube for 60 hours. Within 48 hours of cessation of therapy, stools were fully formed and no *Cryptosporidium* oocysts were detected. The mechanism of the colostrum action appears to depend on its antibody content, perhaps by interfering with oocyst re-attachment thus breaking the pathogen's life cycle. But, the observations raise the possibility that orally administered hyperimmune bovine immunoglobulin may be useful in immunologically compromised patients including those with hereditary immune deficiency states or those with acquired immunodeficiencies such as patients with AIDS.

If we accept that hyperimmune bovine immunoglobulin can be protective (and perhaps in some instances therapeutic), the next question is whether or not it is a practical option to be applied on a broad scale, i.e., is it feasible and can it be sufficiently economical to be useful in lesser developed countries. There is no direct information available to answer this question. However, some assumptions can be made based upon published information. For example, the study of *Tacket* et al. (1988), cited above, reported that adult American volunteers were protected against challenge with 10^9 viable enterotoxigenic *E. coli* which was a 100% infective dose in control subjects. The protected volunteers were given 3.55 g of milk immunoglobulin concentrate (MIC) three times a day for seven days. The MIC used contained 40% immunoglobulin and a mixture of antibodies, of which many may be assumed to be irrelevant. If we assume, e.g., that 10% of the antibodies were effective then the dose of effective immunoglobulin per volunteer per day was of the order of 400 mg. We may also assume that this dose was excessive in view of the fact that it resulted in 100% protection against an unnaturally high challenge. Can we assume that 10-fold less, i.e., 40 mg per day, would be sufficient? Although that remains to be shown by appropriate experimental studies, it seems to us to be a potentially acceptable assumption. In as much as a kilogram or more of immunoglobulin can be harvested from a newly parturient cow, based on the above assumptions, a single cow could provide 25,000 daily doses for adults and perhaps 100,000 doses for children. These assumptions are related to using colostrum from newly parturient cows. It is also possible that there could be sufficient antibody present in mature milk from immunised cows to be protective. Further, herds of cows could potentially be immunised with multiple antigens to provide milk which could be protective against a variety of pathogens.

Admittedly, the problem of providing antibody-containing infant feeding formulae or the antibody itself in a stable and sanitary form, remains to be resolved. But, in as much as infants who are not breast-fed are presently being fed breast-milk substitutes or formulae in an unsanitary way, it seems that the provision of protective formulae would be an improvement.

We conclude that bovine lactogenic immunity is indeed a concept whose time has come. Or, at least, the time has come for further evaluation of the concept.

ACKNOWLEDGEMENTS

Studies in the authors' laboratory, reported herein, were supported in part by grants AI 16776 and AI 17312 from the National Institute of Allergy and Infectious Diseases. The authors are grateful to Mohammad Kazemi for the checkerboard immunoblotting of the bovine colostral antitoxic immunoglobulin.

LITERATURE

Apple, R.D.: Mothers and medicine: A social history of infant feeding 1890-1950. The University of Wisconsin Press, Madison (1987).

Barnes, G.L., Hewson, P.H., McClellan, J.A., Doyle, L.W., Knoches, A.M.L., Kitchen, W.H., and Bishop, R.F.: A randomized trial of oral gammaglobulin in low-birth-weight infants infected with rotavirus. Lancet i, 1371-1373 (1982).

Boesman-Finkelstein, M. and Finkelstein, R.A.: Antimicrobial activities of human milk: Inhibitory activity on enteric pathogens. FEMS Microbiol. Lett. 27, 167-174 (1985).

Boesman-Finkelstein, M. and Finkelstein, R.A.: Passive oral immunisation of children. Lancet, ii, 1336 (1989).

Boesman-Finkelstein, M., Walton, N.E., and Finkelstein, R.A.: Bovine lactogenic immunity against cholera toxin-related enterotoxins and *Vibrio cholerae* outer membranes. Infect. Immun. 57, 1227-1234 (1989).

Brussow, H., Hilpert, H., Walther, I., Sidoti, J., Mietens, C., and Bachman, P.: Bovine milk immunoglobulins for passive immunity to infantile rotavirus gastroenteritis. J. Clin. Microbiol. 25, 982-986 (1987).

Cleason, M. and Merson, M.H.: Global progress in the control of diarrheal diseases. Pediat. Infect. Dis. J. 9, 345-355 (1990).

Davidson, G.P., Daniels, E., Nunan, H., Moore, A.G., Whyte, P.B.D., Franklin, K., McCloud, P.I., and Moore, D.J.: Passive immunization of children with bovine colostrum containing antibodies to human rotavirus. Lancet ii, 709-712 (1989).

Dolan, S.A., Boesman-Finkelstein, M., and Finkelstein, R.A.: Antimicrobial activity of human milk against pediatric pathogens. J. Infect. Dis. 154, 722-725 (1986).

Dolan, S.A., Boesman-Finkelstein, M., and Finkelstein, R.A.: Inhibition of enteropathogenic bacteria by human milk *in vitro*. Pediat. Infect. Dis. J. 8, 430-436 (1989).

Ebina, T., Sato, A., Umeza, K., Ishida, H., Ohyama, S., Oizumi, A., Aikawa, K., Kitagiri, S., Katsushima, N., Imai, A., Kitaoka, S., Suzuki, H., and Konno, T.: Prevention of rotavirus infection by oral administration of cow colostrum containing anti-human-rotavirus-antibody. Med. Microbiol. Immunol. 174, 177-185 (1985).

Ehrlich, P.: Experimentelle untersuchungen über immunität. Deutsche Medicin. Wochenschrift 17, 1218-1219 (1891).

Ehrlich, P.: Über immunität durch vererbung und saugung. Ztschr. Hyg. Infektionskr. 12, 183-203 (1892).

Eibl, M.M., Wolf, H.M., Furnkranz, H., and Rosenkranz, A.: Prevention of necrotizing enterocolitis in low-weight infants by IgA-IgG Feeding. N. Engl. J. Med. 319, 1-7 (1988).

Feachem, R.G. and Koblinsky, M.A.: Interventions for the control of diarrhoeal diseases among young children: Promotion of breast-feeding. Bull. W.H.O. 62, 271-291 (1984).

Fildes, V.: Breast, bottles, and babies. Edinburgh University Press, Edinburgh (1986).

Goldman, A.S., Ham Pong, A.J., and Goldblum, R.A.: Host defenses: Development and maternal contributions. Adv. Pediat. 32, 71-100 (1985).

Grant, J.P.: The State of the World's Children 1990. Oxford University Press (Published for UNICEF), New York, p. 25 (1990).

Hanson, L.A.: Comparative immunological studies of the immunoglobulins of human milk and blood serum. Int. Arch. Allergy Appl. Immunol. 18, 241-267 (1961).

Hanson, L.A., Carlsson, B., Jahil, F., Hahn-Zoric, M., Hermodson, S., Karlberg, J., Mellander, L., Kahn, S.R., Lindblad, B., Thiringer, K., and Zaman, S.: Antiviral and antibacterial factors in human milk. In: Biology of milk (Ed. Hanson, L.A.). Nestlé Nutrition Workshop Series 15, 141-157 (1988).

Hilpert, H., Brussow, H., Mietens, C., Sidoti, J., Lerner, L., and Werchau, H.: Use of bovine milk concentrate containing antibody to rotavirus to treat rotavirus gastroenteritis in infants. J. Infect. Dis. 156, 158-166 (1987).

Hilpert, H.H., Gerber, H., Depeyer, E., and Nussle, D.: Gastrointestinal passage of bovine anti-*E. coli* milk immunoglobulins (Ig) in infants. Nestlé Research News, 134-138 (1974/1975).

Jason, J.M., Nieburg, P., and Marks, J.S.: Mortality and infectious disease associated with infant-feeding practices in developing countries. Pediatrics 74 (Suppl.), 702-727 (1984).

Jelliffe, D.B. and Jelliffe, E.F.P.: Human milk in the modern world. Oxford Medical Publications. Oxford University Press, Oxford (1988).

Kazemi, M. and Finkelstein, R.A.: Checkerboard immunoblotting (CBIB): An efficient, rapid, and sensitive method of assaying multiple antigen/antibody cross-reactivities. J. Immunol. Methods 128, 143-146 (1990).

Kenny, J.F., Boesman, M.I., and Michaels, R.H.: Bacterial and viral coprantibodies in breast-fed infants. Pediatrics 39, 202-213 (1967).

Knodel, J.: Breast feeding and population growth. Science 198, 1111-1115 (1977).

Kovar, M.G., Serdula, M.K., marks, J.S., and Fraser, D.W.: Review of the epidemiologic evidence for an association between infant feeding and infant health. Pediatrics 74 (Suppl.), 615-638 (1984).

Larguia, A.M., Urman, J., Stoliar, O.A., Ceriani, J.M., O'Donnell, A., Buscaglia, J.C., and Martinez, J.C.: Fresh human colostrum for the prevention of *E. coli* diarrhea. A clinical experience. J. Trop. Pediat. Environ. Child Health 23, 289-290 (1977).

Ladcelles, A.K. and McDowell, G.H.: Localized humoral immunity with particular reference to ruminants. Transplant. Rev. 19, 208-209 (1974).

Lawrence. R.A.: Breastfeeding in modern medicine. In: Breastfeeding: a guide for the medical profession. Third Edition. Mosby, St. Louis (1989).

Lindh, E.: Increased resistance of immunoglobulin dimers to proteolytic degradation after binding of secretory component. J. Immunol. 114, 284-290 (1975).

Marquardt, M.: Paul Ehrlich. Henry Schuman, New York (1951).

Mata, L.: Breast-feeding: Main promoter of infant health. Am. J. Clin Nutr. 31, 2058-2065 (1978).

Mata, L.: Breast-feeding and host defense. Frontiers Gastrointest. Res. 13, 119-133 (1986).

May, J.T.: Microbial contaminants and antimicrobial properties of human milk. Microbiol. Sci. 5, 42-46 (1988).

McClead, R.E. and Gregory, S.A.: Resistance of bovine colostral anti-cholera toxin antibody to *in vitro* and *in vivo* proteolysis. Infect. Immun. 44, 474-478 (1984).

McClead, R.E., Butler, T., and Rabbani, G.H.: Orally administered bovine colostral anticholera toxin antibodies: Results of two clinical trials. Am. J. Med. 85, 811-816 (1988).

Mietens, C., Kleinhorst, H., Hilpert, H., Gerber, H., Amster, H., and Pahud, J.J.: Treatment of infantile *E. coli* gastroenteritis with specific bovine anti-*E. coli* milk immunoglobulins. Eur. J. Pediatr. 132, 239-252 (1979).

Narayanan, I., Bala, S., Prakash, K., Verma, R.K., and Gujral, V.V.: Partial supplemen-

tation with expressed breast-milk for prevention of infection in low-birth-weight infants. Lancet ii, 561-563 (1980).

Peterson, W.E. and Campbell, B.: Use of protective principles in milk and colostrum in prevention of disease in man and animals. Lancet 75, 494-496 (1955).

Ruiz-Palacios, G.M., Calva, J.J., Pickering, L.K., Lopez-Vidal, Y., Volkow, P., Pezzarossi, H., and West, M.S.: Protection of breast-fed infants against *Campylobacter* diarrhea by antibodies in human milk. J. Pediatr. 116, 707-713 (1990).

Snyder, J.D. and Merson, M.H.: The magnitude of the global problem of acute diarrhoeal disease: A review of active surveillance data. Bull. W.H.O. 60, 605-613 (1982).

Svirsky-Gross, S.: Pathogenic strain of *E. coli* (O111:B4) among prematures and the use of human milk in controlling the outbreak of diarrhea: Ann. Paediatr. 190, 109-115 (1958).

Tacket, C.O., Losonsky, G., Link, H., Hoang, Y., Guesry, P., Hilpert, H., and Levine, M.M.: Protection by milk immunoglobulin concentrate against oral challenge with enterotoxigenic *Escherichia coli*. N. Eng. J. Med. 318, 1240-1243 (1988).

Tassovatz, B. and Kotsitch, A.: La lait de femme et son action de protection contre les infections intestinales chez le nouveaune. Ann. Pediatrie 8, 285-288 (1961).

Tzipori, S., Roberton, D., and Chapman, C.: Remission of diarrhoea due to cryptosporidiosis in an immunodeficient child treated with hyperimmune bovine colostrum. British Med. J. 293, 1276-1277 (1986).

Tzipori, S., Roberton, D., Cooper, D., and White, L.: Chronic *Cryptosporidium*-associated diarrhoea and hyperimmune cow colostrum. Lancet ii, 344-345 (1987).

Ungar, B.L.P., Ward, D.J., Fayer, R., and Quinn, C.A.: Cessation of *Cryptosporidium*-associated diarrhea in an acquired immunodeficiency syndrom patient after treatment with hyperimmune bovine colostrum. Gastroenterology 98, 486-489 (1990).

Walker-Smith, J.: St. Bartholomew's hospital in the middle ages. Mod. Med. 45, 39-47 (1975).

Old Herborn University Seminar Monograph 4:
Host-microflora interactions in the first years after birth.
Editors: Peter J. Heidt, Rial D. Rolfe, Volker Rusch, and Dirk van der Waaij.
Institute for Microbiology and Biochemistry, Herborn-Dill, Germany: 33-40 (2002).

INFLUENCE OF INFANT DIETS ON THE ECOLOGY OF THE INTESTINAL TRACT OF HUMAN FLORA ASSOCIATED GNOTOBIOTIC MICE

DAVID J. HENTGES[1], WALLACE W. MARSH[1], BRYON W. PETSCHOW[2], WENDY R. THAL[1], and MELODY K. ADAMS[1]

[1]Department of Microbiology, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA, and [2]Bristol-Myers Company, U.S. Nutritional Group, Evansville, IN 47721, USA

SUMMARY

Germfree mice were associated orogastrically with predominant aerobic and anaerobic intestinal flora components isolated from the faeces of breast-fed human infants. The flora components colonised the intestines of mice and persisted at fixed population levels. Groups of flora associated mice received diets consisting of either human milk, cow's milk, Enfamil or Enfamil formula modifications exclusively for two weeks. The effects of the diets on small intestinal and caecal flora composition, caecal pH and resistance to intestinal colonisation with *Salmonella typhimurium* were then determined. Total populations of both aerobes and anaerobes were greatest in mice fed human milk. *Bifidobacterium* and *Bacteroides* were the predominant anaerobes and *Enterococcus* the predominant aerobe. Similarly, *Bifidobacterium* and *Enterococcus* predominated in the intestines of Enfamil-fed mice. Modification of the Enfamil formula did not have a significant effect on flora composition. In mice receiving cow's milk, however, *Bacteroides* and Gram-negative enteric bacteria were the predominant intestinal bacteria. The pH of caecal contents was lowest in mice consuming human milk and highest in mice consuming cow's milk. Titration of the diets demonstrated that human milk had the least buffering capacity and cow's milk the greatest. After orogastric challenge, mice consuming human milk were significantly more resistant to colonisation with *S. typhimurium* than mice consuming cow's milk, Enfamil or Enfamil formula modifications.

INTRODUCTION

A number of studies demonstrate that diet influences the composition of the intestinal flora of human infants. In breast-fed infants, bifidobacteria predominate whereas in formula-fed infants other anaerobes, in addition to bifidobacteria, and facultatively anaerobic bacteria are all present at high population levels (*Benno* et al., 1984; *Mevissen-Verhage* et al., 1985a, 1985b; *Stark* and *Lee*, 1982). The pH of the faeces of breast-fed infants, at a mean of 5.0 to 5.5, is considerably lower than the pH of the faeces of formula-fed infants, at a mean of 8.0 to 9.0 (*Bullen* et al., 1977; *Cooperstock* and *Zedd*, 1983;

Table 1: Composition of the inoculum given to mice

Bifidobacterium bifidum*	10^7
Bacteroides sp.	10^6
Clostridium bifermentans	10^4
Enterococcus faecalis	10^4
Escherichia coli	10^5
Staphylococcus epidermidis	10^2

*Streptomycin resistant variant

Willis et al., 1973).

The data indicate that human milk fa-vours multiplication of bifidobacteria in the intestine of infants. It contains a greater amount of lactose and has less buffering capacity than cow's milk (*Bullen* and *Willis*, 1971). The low intestinal pH that is induced by human milk, enhances multiplication of bifidobacteria (*Stark* and *Lee*, 1982) but specific growth promoting factors for bifidobacteria present in human milk may also be important (*Beerens* et al., 1980). Some researchers speculate that differences in intestinal flora composition account, in part, for the greater resistance of breast-fed infants to enteric infections compared to formula-fed infants (*Beerens* et al., 1980; *Kovar* et al., 1985). Bifidobacteria may contribute to this protective capacity by establishing an acetate buffer in the intestinal tract of breast-fed infants (*Bullen* and *Tearle*, 1976).

The purpose of this project is to examine the influence of various human infant diets on the ecology of the intestinal tract employing an experimental animal model. The composition of the intestinal flora of conventional animals is sufficiently different from that of the human infant to preclude the use of these animals for this purpose. We have therefore associated germfree mice with predominate flora components isolated from the faeces of breast-fed human infants and examined the influence of milk diets, infant formulas and formula modifications on the ecology of the intestinal tract.

MATERIALS AND METHODS

Flora analysis

Germfree BALB/c mice were housed in Trexler type isolators and were given sterile fortified rodent chow (Ralston Purina Co., St. Louis, MO) and sterile water *ad libitum*. Mice used in the experiments, weighing approximately 25 g, were randomly selected and transferred to another isolator where they were associated orogastrically with 0.1 ml of a suspension containing a mixture of flora components isolated from the faeces of breast-fed human infants (Table 1). They were then transferred to barrier isolators, which permitted exposure to laboratory personnel but not to other animals. In the barrier isolators the mice were separated into groups of five each, and were given sterile water and either human milk, cow's milk, Enfamil low iron formula (Mead Johnson Nutritionals, Evansville, IN) or a modification of the Enfamil formula exclusively for 14 days. Human milk was obtained from healthy volunteers who were not receiving antibiotics or other medications at the time of donation. Formula modification consisted of the addition of sterilised hog gastric mucin to

Table 2: Media used for isolation of intestinal anaerobic bacteria

Medium	Predominant organism isolated	Incubation time (days)
Brucella blood*	Total anaerobes	5
Bacteroides bile aesculin	Bacteroides	2
Cycloserine - mannose	Clostridium	2
Reinforced clostridial agar with streptomycin	Bifidobacterium	2

*Enriched with vitamin K

Enfamil at a concentration of 0.25 mg/ml (Enfamil with mucin) and the alteration of Enfamil to contain a high casein to whey protein ratio (Formula 3305) rather than the high whey protein to casein ratio present in Enfamil.

On day 14, the mice were removed from the barrier isolators, sacrificed by cervical dislocation, and introduced into an anaerobic chamber. Inside the chamber the small intestines and caeca of the mice were aseptically removed. The organs were weighed and then homogenised in 9 volumes of prereduced sterile 0.05% yeast extract. Serial 100-fold dilutions of the homogenates were plated on various prereduced selective anaerobic media (Table 2). The plates were incubated anaerobically at 37°C for 48 hours. The dilution series was removed from the anaerobic chamber and plated on various selective aerobic media (Table 3). These plates were incubated aerobically for 48 hours at 37°C. Colonies of aerobic and anaerobic organisms were counted and the organisms were identified by standard bacteriological procedures including Gram-staining and API analysis. Counts were reported as viable organisms per gram homogenate.

pH Analysis

On day 14 after flora association, mice were sacrificed by cervical dislocation and introduced into the anaerobic chamber. The caeca were exposed and a small incision made in the wall of each. A micro combination pH electrode (Microelectrodes Inc. Londonerry, NH) was inserted through the incision into the luminal contents. pH values were obtained using a Corning 125 Potentiometer.

Buffering Capacity

Twenty ml aliquots of human milk, cow's milk, Enfamil or Enfamil formula modifications were each titrated with 0.1N NaOH and 0.1N HCl. Titration curves were plotted, obtained by the addition of small increments of either acid or base. The pH values were determined using a combination electrode and a Corning 125 Potentiometer.

Table 3: Media used for isolation of intestinal aerobic bacteria

Medium	Predominant organism isolated	Incubation time (days)
Trypticase soy blood	Total aerobes	2
MacConkey	E. coli and other enterobacteria	1-2
Bile aesculin azide	Enterococcus	2

Table 4: Effect of diet on caecal flora composition

	Human milk	Cow's milk	Enfamil
Total anaerobes	10.65 ± 0.28[1]	10.03 ± 0.36	10.08 ± 0.21
Clostridium	4.79 ± 1.61 (4/5)[2]	4.33 ± 1.31 (4/5)	3.31 ± 0.21 (3/5)
Bacteroides	9.31 ± 0.54	8.71 ± 0.74	7.79 ± 1.15
Bifidobacterium	9.33 ± 0.50	7.89 ± 0.79	8.64 ± 0.61
Total aerobes	9.85 ± 0.19	9.06 ± 0.14[3]	9.12 ± 0.18[3]
Enterobacteriacae	8.74 ±0.32	8.69 ± 0.22	8.67 ± 0.21
Enterococcus	9.43 ± 0.21	7.89 ± 0.27[3]	8.79 ± 0.20

[1]Mean log 10 viable bacteria/gram ± SEM.
[2]Incidence of isolation of the organism from the mice; in all other cases it was 5/5.
[3]Statistically significant fewer counts ($p<0.05$) than from mice receiving human milk.

Colonisation Resistance

The effects of consumption of the various diets by the gnotobiotic mice on colonisation resistance against *Salmonella typhimurium* were determined. Fourteen days after association with human infant flora components (Table 1) five mice on each of the diets were challenged orogastrically with 0.1 ml of a suspension of 1.0×10^4 streptomycin resistant *S. typhimurium*. Three days after challenge, the mice were sacrificed by cervical dislocation and the small intestines and caeca were removed aseptically, were weighed and were homogenised individually in 9 volumes of 0.05% yeast extract. Serial 100-fold dilutions of the homogenates were plated on Mac Conkey's Agar containing 1mg/ml streptomycin sulphate, which is selective for the streptomycin-resistant *S. typhimurium* strain. The plates were incubated aerobically for 24 hours at 37°C. Colony counts were reported as viable organisms per gram homogenate. Each experiment was repeated twice.

Statistical Analysis

Statistical evaluations of the significance of the differences in viable bacterial counts obtained from intestinal homogenates and pH values of caecal contents were performed using Fisher's least significant difference test and the Duncan-Neuman-Keul test at the 95% confidence interval level.

RESULTS

Results of studies comparing the effects of exclusive consumption by the mice of human milk, cow's milk, Enfamil or Enfamil formula modifications on the ecology of the gastrointestinal tract will be described.

Initially, the effects of diets consisting of either human milk, cow's milk or Enfamil were examined. Flora analysis of caecal homogenates demonstrated that, with all diets, anaerobes outnumbered aerobes by a factor of approximately 10 to 1 and that bacterial populations were greater in mice consuming human milk than in mice consuming cow's milk or Enfamil (Table 4). In mice consuming human milk, *Bifidobacterium* and *Bacteroides* were the predominant anaerobes and *Enterococcus* was the predominant aerobe. Similarly, *Bifidobacterium* and *Enterococcus* predominated in the

Table 5: Effect of diet on small intestinal flora composition

	Human milk	Cow's milk	Enfamil
Total anaerobes	8.86 ± 0.10[1]	7.95 ± 0.43[3]	7.93 ± 0.12[3]
Clostridium	2.55 ± 1.25 (3/5)[2]	2.98 ± 1.04 (4/5)	1.96 ± 1.28 (3/5)
Bacteroides	7.39 ± 0.73	6.69 ± 0.43	5.68 ± 0.64
Bifidobacterium	7.17 ± 0.18	6.19 ± 0.52	6.85 ± 0.48
Total aerobes	8.39 ± 0.19	7.15 ± 0.16[3]	7.64 ± 0.10[3]
Enterobacteriacae	7.62 ± 0.18	6.59 ± 0.22[3]	6.80 ± 0.21[3]
Enterococcus	7.90 ± 0.15	6.56 ± 0.22[3]	7.14 ± 0.23[3]

[1]Mean log 10 viable bacteria/gram ± SEM.
[2]Incidence of isolation of the organism from the mice; in all other cases it was 5/5.
[3]Statistically significant fewer counts ($p<0.05$) than from mice receiving human milk.

intestinal tract of Enfamil-fed mice. *Bifidobacterium* populations were greatest in mice consuming human milk and smallest in mice consuming cow's milk, although the difference was not statistically significant. In mice consuming cow's milk, *Bacteroides* and Gram-negative enteric bacteria predominated. Similar results were obtained when small intestinal homogenates were analysed except that bacterial counts were approximately 100-fold lower than counts from caecal homogenates (Table 5). In several instances, counts obtained from mice consuming human milk were significantly greater than counts obtained from mice consuming either cow's milk or Enfamil.

Subsequent experiments were done examining the effects of modifications of the Enfamil formula on the composition of the intestinal flora of the mice. Controls with each experiment consisted of groups of mice consuming human milk and mice consuming Enfamil. Diets of Enfamil with mucin or Formula 3305 (high casein to whey protein ratio) had no effects on the composition of the flora. There were no statistically significant differences in counts of any of the bacteria isolated from the caecum or the small intestine of the mice consuming human milk, Enfamil, or Enfamil formula modifications in these experiments.

Table 6: Influence of diet on the pH of caecal contents

	Diet	pH
Study 1:	Human milk	5.99 ± 0.11
	Enfamil	6.88 ± 0.11[1]
	Cow's milk	7.16 ± 0.06[1]
Study 2:	Human milk	5.86 ± 0.09
	Enfamil	6.35 ± 0.14
	Enfamil with mucin	6.73 ± 0.23[1]
Study 3:	Human milk	6.20 ± 0.15
	Enfamil (liquid)	7.02 ± 0.11[1]
	Formula 3305	6.56 ± 0.22

[1]Statistically significantly greater values ($p<0.05$) than from mice consuming human milk.

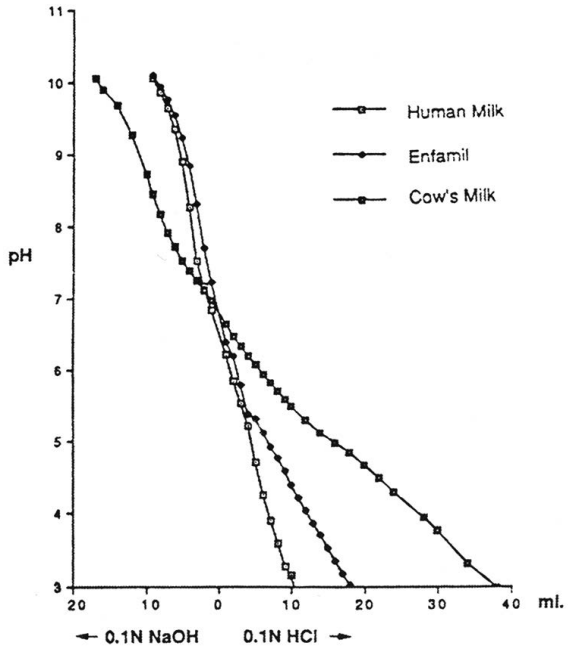


Figure 1: Titration curve of various diets.

The influence of the various diets on the pH of the caecal contents of the mice was also determined. Results are illustrated in Table 6. In all three studies, the mean pH of caecal contents of mice consuming human milk was lower than the pH of caecal contents of mice consuming the other diets. In several cases these differences were statistically significant. However, there were no significant differences in the pH of the contents between mice consuming cow's milk, Enfamil or Enfamil formula modifications. Contents of mice consuming cow's milk had the highest pH, which was more than one log10 greater than the pH of contents of mice consuming human milk.

Human milk had considerably less buffering capacity than cow's milk and somewhat less buffering capacity than Enfamil or Enfamil formula modifications. Figure 1 shows that approximately 7.0 ml of 0.1N HCl was required to lower the pH of human milk from 7.0 to 4.0 and 12.0 ml and 28.0 ml were required to lower than pH of Enfamil and cow's milk, respectively, to the same degree. Modifications of the Enfamil formula had no appreciable effect on its buffering capacity.

The colonisation resistance of the gnotobiotic mice on the various diets to challenge with *S. typhimurium* was determined next. Results are presented in Table 7. The table shows that there were significantly fewer *S. typhimurium* isolated from caecal homogenates of mice consuming human milk than mice consuming cow's milk, Enfamil or Enfamil formula modifications. However, counts obtained from mice consuming cow's milk were not significantly different from counts obtained from mice consuming Enfamil. Nor did modifications of the Enfamil formula significantly alter caecal *S. typhimurium* counts when compared with a diet of Enfamil.

Table 7: Influence of diet on population levels of *S. typhimurium* in the mouse caecum and the incidence of colonisation

	Diet	pH	
Study 1:	Human milk	5.48 ± 0.40	14/15
	Enfamil	6.55 ± 0.24[1]	15/15
	Cow's milk	6.71 ± 0.37[1]	15/15
Study 2:	Human milk	5.32 ± 0.44	13/13
	Enfamil	7.96 ± 0.39[1]	13/13
	Enfamil with mucin	7.60 ± 0.24[1]	15/15
Study 3:	Human milk	5.22 ± 0.52	4/5
	Enfamil (liquid)	8.45 ± 0.11[1]	5/5
	Formula 3305	8.83 ± 0.23[1]	5/5

[1]Statistically significantly greater values ($p<0.05$) than from mice consuming human milk.

DISCUSSION

These studies demonstrate that germfree mice can be associated successfully with intestinal flora components isolated from the faeces of breast-fed human infants. The flora components colonise the small and large intestines of the animals and persist at fixed population levels.

Dietary variations influenced the composition of the intestinal flora of the mice to a moderate degree, although differences in population levels of bacteria were usually not statistically significant. *Bifidobacterium*, generally, was the predominant anaerobe and *Enterococcus* the predominant aerobe isolated from mice consuming human milk or Enfamil. *Bacteroides* and Gram-negative enteric bacteria predominated, on the other hand, in the intestines of mice consuming cow's milk. These results are similar to those reported in other studies demonstrating the predominance of *Bifidobacterium* in the intestinal tract of human breast-fed infants (*Benno* et al., 1984; *Mevissen-Verhage* et al., 1985a, 1985b; *Stark* and *Lee*, 1982). When compared with a diet of Enfamil, Enfamil formula modifications had little effect on the composition of the intestinal flora of the mice.

A consistent finding in all of our studies was the lower pH of the caecal contents of mice consuming human milk than of mice consuming cow's milk, Enfamil or Enfamil formula modifications. This is in accord with the results of studies showing that the pH of the faeces of human breast-fed infants is lower than the pH of the faeces of formula-fed infants (*Bullen* et al., 1977; *Cooperstock* and *Zedd*, 1983; *Willis* et al., 1973). The reason for the lower pH of intestinal contents when human milk is consumed is unknown. However, we demonstrated in these studies that the buffering capacity of human milk is much less than the buffering capacity of cow's milk and somewhat less than the buffering capacity of the infant formulas. The pH of the intestinal contents may therefore be a reflection of the buffering capacities of the diets.

Human milk consumption by the mice provided significantly greater protection against colonisation with *S.*

typhimurium than consumption of cow's milk, Enfamil or Enfamil formula modifications. Greater protection may be a consequence of low intestinal pH which results in increased concentrations of undissociated fatty acid molecules that inhibit multiplication of *Salmonella* and other enteric pathogens (*Hentges*, 1983). Alternatively, it may be due to some other factor such as the presence of protective anti-*Salmonella* antibodies in human milk. Additional work needs to be done to determine the mechanisms responsible for the protection that is apparent when human milk is consumed.

LITERATURE

Beerens, H., Romond, C., and Neut, C.: Influence of breast-feeding on the bifid flora of the new-born intestine. Am. J. Clin. Nutr. 33, 2434-2439 (1980).

Benno, Y., Sawada, K., and Mitsuoka, T.: The intestinal microflora of infants: Composition of fecal flora in breast-fed and bottle-fed infants. Microbiol. Immunol. 28, 975-986 (1984).

Bullen, C.L. and Tearle, P.V.: Bifidobacteria in the intestinal tract of infants: An *in vitro* study. J. Med. Microbiol. 9, 335-344 (1976).

Bullen, C.L., Tearle, P.V., and Stewart, M.G.: The effect of "humanised" milks and supplemented breast feeding on the faecal flora of infants. J. Med. Microbiol. 10, 403-413 (1977).

Bullen, C.L. and Willis, A.T.: Resistance of the breast-fed infant to gastroenteritis. Brit. Med. J. 3, 338-343 (1971).

Cooperstock, M.S. and Zedd, A.J.: Intestinal flora of infants. In: Human intestinal microflora in health and disease (Ed.: Hentges, D.J.). Academic Press, New York, 79-99 (1983).

Hentges, D.J.: Role of the intestinal microflora in host defense against infection. In: Human intestinal microflora in health and disease (Ed.: Hentges, D.J.). Academic Press, New York, 311-331 (1983).

Kovar, M.G., Serdula, M.K., Marks, J.S., and Fraser, D.W.: Review of the epidemiologic evidence for an association between infant feeding and infant health. Pediatrics 74 (suppl.), 615-638 (1985).

Mevissen-Verhage, E.A.E., Marcelis, J.H., Harmsen-van Amerongen, W.C.M., de Vos, N.M., Berkel, J., and Verhoef, J.: Effect of iron on neonatal gut flora during the first week of life. Eur. J. Clin. Microbiol. 4, 14-18 (1985a).

Mevissen-Verhage, E.A.E., Marcelis, J.H., Harmsen-van Amerongen, W.C.M., de Vos, N.M., and Verhoef, J.: Effect of iron on neonatal gut flora during the first three months of life. Eur. J. Clin. Microbiol. 4, 273-278 (1985b).

Stark, P.L. and Lee, A.: The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. J. Med. Microbiol. 15, 189-203 (1982).

Willis, A.T., Bullen, C.L., Williams, K., Flagg, C.G., Bourne, A., and Vignon, M.: Breast milk substitute: a bacteriological study. Brit. Med. J. 4, 67-72 (1973).

Old Herborn University Seminar Monograph 4:
Host-microflora interactions in the first years after birth.
Editors: Peter J. Heidt, Rial D. Rolfe, Volker Rusch, and Dirk van der Waaij.
Institute for Microbiology and Biochemistry, Herborn-Dill, Germany: 41-60 (2002).

THE DEVELOPMENT OF COLONISATION RESISTANCE IN THE INFANT

RIAL D. ROLFE

Department of Microbiology, School of Medicine,
Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA

SUMMARY

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

INTRODUCTION

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

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

DEVELOPMENT OF INTESTINAL FLORA

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

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

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

Table 1: Mechanisms of colonization resistance

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

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

MECHANISMS OF COLONISATION RESISTANCE

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

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

Influence of host on microbial flora

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

Receptors on intestinal mucosa

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

Antimicrobial agents

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

Diet

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

Immunologic factors

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

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

Bacterial antagonism

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

Bile acids

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

Depletion of or competition for essential substrates

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

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

Competition for bacterial receptor sites

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

Creation of a restrictive physiologic environment

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

Hydrogen ion concentration

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

Oxidation-reduction potential

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

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

Volatile fatty acids

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

Elaboration of an antibiotic-like substance

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

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

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

DEVELOPMENT OF COLONISATION RESISTANCE AGAINST *CLOSTRIDIUM DIFFICILE*

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

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

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

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

Antibiotic treatment

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

Infant colonisation

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

Germfree animals

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

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

***In vitro* inhibition**

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

lactobacilli and group D enterococci displaying the most antagonistic activity.

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

In vivo inhibition by faecal homogenates

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

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

Inhibition by non-toxogenic strains of *Clostridium difficile*

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

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

CLOSTRIDIUM DIFFICILE COLONISATION OF INFANT HAMSTERS

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

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

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

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

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

Asymptomatic colonisation

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

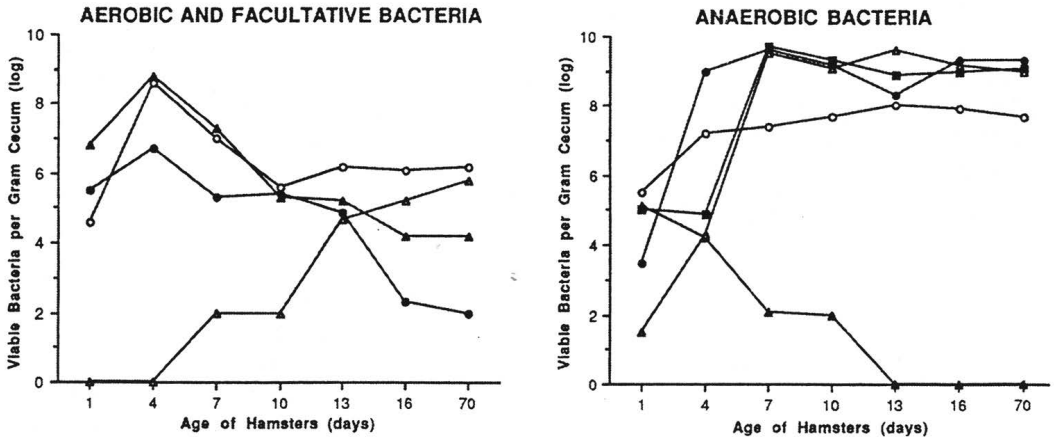


Figure 1: Development of the aerobic, facultative and anaerobic caecal flora of hamsters. (Adapted from *Rolfe*, 1984).
Three hamsters at each age were sacrificed, and the aerobic, facultative and anaerobic caecal flora was determined with selective and non-selective media. The results are depicted as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

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

Development of normal intestinal flora

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

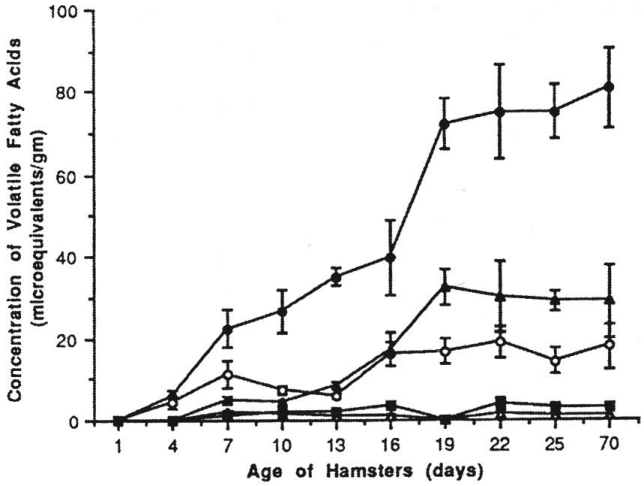


Figure 2: Concentrations of volatile fatty acids in the caeca of hamsters as determined by gas-liquid chromatography. (Adapted from *Rolfe*, 1984).
The values are mean concentrations (micro-equivalents per gram of caecal content [wet weight]) + standard deviation of volatile fatty acids for five pools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

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

Role of volatile fatty acids

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

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

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

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

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

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

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

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

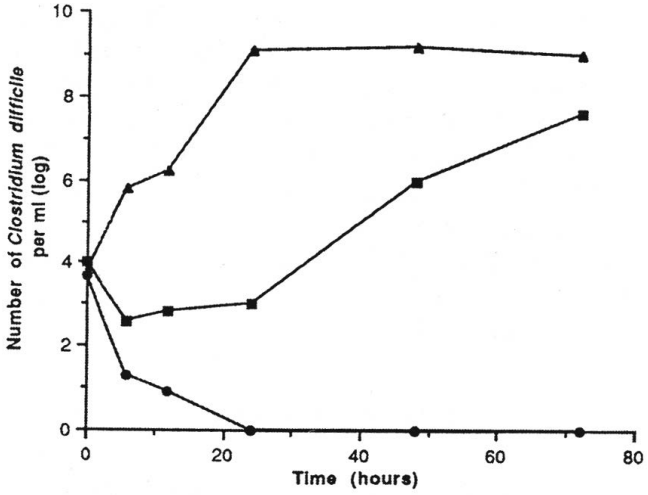


Figure 3: Growth of *Clostridium difficile* in broth containing mixtures of volatile fatty acids at average concentrations present in the caecal of hamsters at different ages. (Adapted from *Rolfe*, 1984).
The pH of the volatile fatty acid mixtures was adjusted to correspond to the average pH measure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

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

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

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

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

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

CONCLUSION

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

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

ACKNOWLEDGEMENTS

This work was supported, in part, by Public Health Service grant RO1-AI123894 from the National Institute of Allergy and Infectious Disease.
We thank Woosun Song for excellent technical assistance during the course of these investigations.

LITERATURE

Aly, R. and Shinefield, H.R. Bacterial Interference. CRC Press, Boca Raton, 1-183 (1982).

Arnon, S.S.: Infant botulism. Annu. Rev. Med. 31, 541-560 (1980).

Barclay, F.E. and Borrielllo, S.P.: *In vitro* inhibition of *Clostridium difficile*. Eur. J. Chemother. Antibiotics 2, 155-156 (1982).

Bennet, R., Erickssen, M., Nord, C.E., and Zetterstrom, R.: Impact of various antibiotics on the fecal flora of newborn infants. Microecol. Ther. 14, 251 (1984).

Benno, Y., Sawada, K., and Mitsuoka, T.: The intestinal microflora of infants: Composition of fecal flora in breast-fed and bottle-fed infants. Microbiol. Immunol. 28, 975-986 (1984).

Bohnhoff, M., Miller, C.P., and Martin, W.R.: Resistance of the mouse intestinal intestinal tract to experimental *Salmonella* infection. I. Factors which interfere with initiation of infection by oral inoculation. J. Exp. Med. 120, 805-816 (1964).

Bonglovanni, A.M.: Bile acid content of gall bladders of infants, children and adults. J. Clin. Endocrinol. 25, 678-685 (1965).

Boriello, S.P. and Barclay, F.E.: Protection of hamsters against *Clostridium difficile* ileocaecitis by prior colonisation with non-pathogenic strains. J. Med. Microbiol. 19, 339-350 (1985).

Boriello, S.P., Barclay, F.E., and welch, A.R.: Evaluation of the predictive capability of an *in vitro* model of colonization resistance to *Clostridium difficile* infection. Microb. Ecol. Hlth. Dis. 1, 61-64 (1988).

Bowden, T.A., Mansberger, A.R., and Lykins, L.E.: Pseudomembranous enterocolitis: Mechanism of restoring flora homeostasis. Am. Surg. 74, 178-183 (1978).

Bullen, C.L., Tearle, P.V., and Stewart, M.G.: The effect of "humanised" milks and supplemented breast feeding on the faecal flora of infants. J. Med. Microbiol. 10, 28-39 (1977).

Byrne, B.M. and Dankert, J.: Volatile fatty acids and aerobic flora in the gastrointestinal tract of mice under various conditions. Infect. Immun. 23, 559-563 (1979).

Carman, R.J. and Boriello, S.P.: Infectious nature of *Clostridium spiroforme*-mediated rabbit enterotoxaemia. Vet. Microbiol. 9, 497-502 (1984).

Caugant, D.A., Levin, B.A., and Selander, R.K.: Genetic diversity and temporal variation in the *E. coli* population of a human host. Genetics 98, 467-490 (1981).

Cheney, C.P. and Boedeker, E.C.: Rabbit mucosal receptors for an enteropathogenic *Escherichia coli* strain: Appearance of bacterial receptor activity at weaning. Gastroenterology 87, 821-826 (1984).

Cooperstock, M.S., Steffen, E., Yolken, R., and Onderdonk, A.: *Clostridium difficile* in normal infants and sudden infant death syndrome: An association with infant formula feeding. Peds. 70, 91-95 (1982).

Cooperstock, M.S. and Zedd, A.J.: Intestinal flora of infants. In: Human intestinal microflora in health and disease. (Ed.: Hentges, D.J.). Academic Press, New York, 79-99 (1983).

Czuprinsky, C.J., Johnson, W.J., Balish, E., and Wilkins, T.: Pseudomembranous colitis in *Clostridium difficile*-monoassociated rats. Infect. Immun. 39, 1368-1376 (1983).

Dabard, J., Dubos, F., Martinet, L., and Ducluzeau, R.: Experimental reproduction of

neonatal diarrhea in young gnotobiotic hares simultaneously associated with *Clostridium difficile* and other *Clostridium* strains. Infect. Immun. 24, 7-11 (1979).

Davidson, J. and Hirsch, D.C.: Use of the K88 antigen for *in vivo* bacterial competition with porcine strains of enteropathogenic *Escherichia coli*. Infect. Immun. 12, 134-136 (1975).

Donta, S.T.: Mechanism of action of *Clostridium difficile* toxins. In: *Clostridium difficile*: Its role in intestinal disease, (Eds.: Rolfe, R.D. and Finegold, S.M.). Academic Press, Inc., New York, 169-181 (1988).

Drasar, B.S. and Hill, M.J.: Human intestinal flora. Academic Press, New York, 1-263 (1974).

Floch, M.H., Binder, J.J., Filburn, B., and Gerhengoren, W.: The effect of bile acids on intestinal microflora. Am. J. Clin. Nutr. 25, 1418-1426 (1972).

Freter, R.: Experimental enteric *Shigella* and *Vibrio* infections in mice and guinea pigs. J. Exp. Med. 104, 411-418 (1956).

Freter, R.: Recapitulation: Interactions among interactions. In: Microbiology-1975. (Ed.: Schlessinger, C.D.). American Society for Microbiology, Washington, D.C., 154-157 (1975).

Freter, R. and Abrams, G.D.: Function of various intestinal bacteria in converting germfree mice to the normal state. Infect. Immun. 6, 119-126 (1972).

Freter, R., Brickner, H., Botney, M., Cleven, D., and Aranki, A.: Mechanisms that control bacterial populations in continuous-flow culture models of mouse large intestinal flora. Infect. Immun. 39, 676-685 (1983).

George, W.L.: Antimicrobial agent-associated diarrhea in adult humans. In: *Clostridium difficile*: Its role in intestinal disease. (Ed.: Rolfe, R.D. and Finegold, S.M.). Academic Press, Inc., New York, 31-44 (1988).

Gorbach, S.L., Nahas, L., and Weinstein, L.: Studies of intestinal microflora. I. Effects of diet, age, and periodic sampling on numbers of fecal microorganisms in man. Gastroenterology 53, 845-855 (1967).

Grutte, F.K., Horn, R., and Haenel, H.: Ernahrung und biochemisch-mikroökologische Vorgange in Enddarm von Sauglingen. Z. Kinderheilkd. 93, 28-39 (1965).

Harris, M.A., Reddy, C.A., and Carter, G.R.: Anaerobic bacteria from the large intestine of mice. Appl. Environ. Microbiol. 31, 907-912 (1976).

Hentges, D.J.: The intestinal flora and infant botulism. Rev. Infect. Dis. 1, 668-671 (1979).

Hentges, D.J.: Role of the intestinal microflora in host defense against infection. In: Human intestinal microflora in health and disease. (Ed.: Hentges, D.J.). Academic Press, Inc., New York, 311-331 (1983).

Huhtanen, C.M.: Bile acid inhibition of *Clostridium botulinum*. Appl. Environ. Microbiol. 38, 216-218 (1979).

Iaconis, J.P. and Rolfe, R.D.: *Clostridium difficile*-associated ileocecitis in clindamycin-treated infant hamsters. Curr. Microbiol. 13, 327-332 (1986).

Ikari, N.S., Kenton, D.M., and Young, V.M.: Interactions in the germfree mouse intestine of colicinogenic and colicin-sensitive microorganisms. Proc. Soc. Exp. Biol. Med. 130, 1280-1284 (1969).

Itoh, K., Lee, W.K., Kawamura, H., Mitsuoka, T., and Magaribuchi, T.: Intestinal bacterial antagonistic to *Clostridium difficile* in mice. Lab. Anim. 21, 20-25 (1987).

Jin, S.W., Bournaud, M., Besnier, M.O., Bourlioux, P., and Fourniat, J.: Transfer of hamster coecal flora to C3H germfree mice: Utilization of this model to study the anti-*Clostridium difficile* microflora. Microecol. Ther. 14, 277-278 (1984).

Lee, A. and Gemmel, E.: Changes in the mouse intestinal microflora during weaning: Role of volatile fatty acids. Infect. Immun. 5, 1-7 (1972).

Lloyd, A.B., Cumming, R.B., and Kent, R.D.: Prevention of *Salmonella typhimurium* infection in poultry by pretreatment of chickens and poults with intestinal extracts. Aust. Vet. J. 53, 82-87 (1977).

Long, S.S. and Swenson, R.M.: Development of anaerobic fecal flora in healthy newborn

infants. J. Pediatr. 91, 298-301 (1977).

Lyerly, D.M. and Wilkins, T.D.: Purification and properties of toxins A and B of *Clostridium difficile*. In: *Clostridium difficile*: Its role in intestinal disease. (Ed.: Rolfe, R.D. and Finegold, S.M.). Academic Press, Inc., New York, 145-167 (1988).

Maier, B.R., Onderdonk, A.B., Baskett, R.C., and Hentges, D.J.: *Shigella*, indigenous flora interactions in mice. Am. J. Clin. Nutr. 25, 1433-1440 (1972).

Malamou-Ladas, H. and Tabaqchali, S.: Inhibition of *Clostridium difficile* by faecal streptococci. J. Med. Microbiol. 15, 569-574 (1982).

Meynell, G.G.: Antibacterial mechanisms of the mouse gut. II. The role of Eh and volatile fatty acids in the normal gut. Brit. J. Expt. Pathol. 44, 209-219 (1963).

Moore, W.E.C. and Holdeman, L.V.: Human fecal flora: The normal flora of 20 Japanese-Hawaiians. Appl. Microbiol. 27, 961-979 (1974).

Mulligan, M.E., Citron, D., Gabay, E., Kirby, B.D., George, W.L., and Finegold, S.M.: Alterations in human fecal flora, including ingrowth of *Clostridium difficile*, related to cefoxitin therapy. Antimicrob. Agents Chemother. 26, 343-346 (1984).

Onderdonk, A.B., Hermos, J.A., and Bartlett, J.G.: The role of the intestinal microflora in experimental colitis. Am. J. Clin. Nutr. 30, 1819-1825 (1977).

Onderdonk, A.B., Cisneros, R.L., and Bartlett, J.G.: *Clostridium difficile* in gnotobiotic mice. Infect. Immun. 28, 277-282 (1980).

Onderdonk, A.B.: Role of the hamster model of antibiotic-associated colitis in defining the etiology of the disease. In: *Clostridium difficile*: Its role in intestinal disease. (Eds.: Rolfe, R.D. and Finegold, S.M.) Academic Press, Inc., New York, 115-125 (1988).

Pongpech, P. and Hentges, D.J.: Inhibition of *Shigella sonnei* and enterotoxigenic *Escherichia coli* by volatile fatty acids in mice. Microb. Ecol. Hlth. Dis. 2, 153-161 (1989).

Raiboud, P., Ducluzeau, R., Dubos, R., Hudault, S., Bewa, H., and Muller, M.C.: Implantation of bacteria from the digestive tract of man and various animals in gnotobiotic mice. Am. J. Clin. Nutr. 33, 2440-2447 (1980).

Rolfe, R.D., Helebian, S., and Finegold, S.M.: Bacterial interference between *Clostridium difficile* and normal fecal flora. J.Infect.Dis. 143, 470-475 (1981).

Rolfe, R.D.: Role of volatile fatty acids in colonization resistance to *Clostridium difficile*. Infect. Immun. 45, 185-191 (1984a).

Rolfe, R.D.: Interactions among microorganisms of the indigenous intestinal flora and their influence on the host. Rev. Infect. Dis. 6, S73-S79 (1984b).

Rolfe, R.D. and Iaconis, J.P.: Intestinal colonization of infant hamsters with *Clostridium difficile*. Infect. Immun. 42, 480-486 (1983).

Rolfe, R.D. and Iaconis, J.P.: *Clostridium difficile* colonization of infant hamsters. In: The influence of antibiotics on the host-parasite relationship II. (Eds.: Adam, D., Hahn, H., and Opferkuch, W.). Springer-Verlag, Berlin, 220-229 (1985).

Rolfe, R.D., Iaconis, J.P., and Kim, P-H.: Symptomatic and asymptomatic colonization of infant hamsters by *Clostridium difficile*. Micrecol. Ther. 16, 217-230 (1986).

Rolfe, R.D.: Asymptomatic intestinal colonization by *Clostridium difficile*. In: *Clostridium difficile*: Its role in intestinal disease. (Eds.: Rolf, R.D. and Finegold, S.M.). Academic Press, Inc., New York, 201-225 (1988).

Salanitro, J.P., Blake, I.G., and Muirhead, P.A.: Isolation and identification of fecal bacteria from adult swine. Appl. Environ. Microbiol. 33, 79-84 (1977).

Savage, D.C.: Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbiol. 31, 107-133 (1977a).

Savage, D.C.: Microbial ecology of the gastrointestinal tract. Ann. Rev. Microbiol. 31, 107-133 (1977b).

Savage, D.C.: Adherence of normal flora to mucosal surfaces. In: Bacterial adherence. (Ed.: Beachey, E.H.). Chapman and Hall, London, 31-60 (1980).

Shwan, A., Sjolin, S., Trottestam, U., and Ar-

onsson, B.: Relapsing *Clostridium difficile* enterocolitis cured by rectal infusion of normal faeces. Scand. J. Infect. Dis. 16, 211-215 (1984).

Seal, D.V., Boriello, S.P., Barclay, F., Welch, A., Piper, M., and Bonnycastle, M.: Treatment of relapsing *Clostridium difficile* diarrhoea by administration of a non-toxigenic strain. Eur. J. Clin. Microbiol. 6, 51-53 (1987).

Shedlofsky, S. and Freter, R.: Synergism between ecologic and immunologic control mechanisms of intestinal flora. J. Infect. Dis. 129, 296-303 (1974).

Smith, H.W. and Crabb, W.E.: The faecal bacterial flora of animals and man: Its development in the young. J. Pathol. Bacteriol. 82, 53-66 (1961).

Stark, P.L. and Lee, A.: The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. J. Med. Microbiol. 15, 189-203 (1982).

Sugiyama, H.: Animal models for the study of infant botulism. Rev. Infect. Dis. 1, 683-687 (1979).

Sullivan, N.M., Mills, D.C., Riemann, H.P., and Arnon, S.S.: Inhibition of growth of *Clostridium botulinum* by intestinal microflora isolated from healthy infants. Microb. Ecol. Hlth. Dis. 1, 179-192 (1988).

Tannock, G.W.: Control of gastrointestinal pathogens by normal flora. In: Current perspectives in microbial ecology. (Eds.: Khug, M.J. and Reddy, C.A.). American Society for Microbiology, Washington, D.C., 374-382 (1984).

Tannock, G.W. and Savage, D.C.: Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract. Infect. Immun. 9, 591-598 (1974).

Tullus, K., Aronsson, B., Marcus, S., and Möllby, R.: Intestinal colonization with *Clostridium difficile* in infants up to 18 months of age. Eur. J. Clin. Microbiol. Infect. Dis. 8, 390-393 (1989).

Tvede, M. and Rask-Madsen, J.: Bacteriotherapy for chronic relapsing *Clostridium difficile* diarrhoea in six patients. Lancet i, 1156-1160 (1989).

van der Waaij, D., Berghuis-de Vries, J.M., and Lekkerkerk-van der Wees, J.E.C.: Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J. Hyg. 69, 405-511 (1971).

Vosbeck, K., handschin, H., Menge, E-B., and Zak, O.: Effects of subminimal inhibitory concentrations of antibiotics on adhesiveness of *Escherichia coli in vitro*. Rev. Infect. Dis. 1, 845-851 (1979).

Wang, Y. and Sugiyama, H.: Botulism in metronidazole-treated conventional adult mice challenged orogastically with spores of *Clostridium botulinum* type A or B. Infect. Immun. 46, 715-719 (1984).

Williams, R.C., Showalter, R., and Kern, F.: *In vivo* effect of bile salts and cholestyramine on intestinal bacteria. Gastroenterol. 69, 483-491 (1975).

Wilson, K.H. and Freter, R.: Interaction of *Clostridium difficile* and *Escherichia coli* with microfloras in continuous-flow cultures and gnotobiotic mice. Infect. Immun 54, 354-358 (1986).

Wilson, K.H. and Perini, F.: Role of competition for nutrients in suppression of *Clostridium difficile* by the colonic microflora. Infect. Immun 56, 2610-2614 (1988).

Wilson, K.H. and Sheagren, J.N.: Antagonism of toxigenic *Clostridium difficile* by nontoxigenic *C. difficile*. J. Infect. Dis. 147, 733-736 (1983).

Wilson, K.H., Silva, J., and Fekety, F.R.: Suppression of *Clostridium difficile* by normal hamster cecal flora and prevention of antibiotic-associated cecitis. Infect. Immun 34, 626-628 (1981).

Wilson, K.H., Sheagren, J.N., Freter, R., Weatherbee, L., and Lyerly, L.: Gnotobiotic models for study of the microbial ecology of *Clostridium difficile* and *Escherichia coli*. J. Infect. Dis. 153, 547-551 (1986).

Old Herborn University Seminar Monograph 4:
Host-microflora interactions in the first years after birth.
Editors: Peter J. Heidt, Rial D. Rolfe, Volker Rusch, and Dirk van der Waaij.
Institute for Microbiology and Biochemistry, Herborn-Dill, Germany: 61-72 (2002).

DEVELOPMENTAL CONTROL OF BACTERIAL RECEPTORS IN THE GASTROINTESTINAL TRACT

SHU-HEH W. CHU and W. ALLAN WALKER

Combined Program in Pediatric Gastroenterology and Nutrition at Harvard Medical School (The Children's Hospital and Massachusetts General Hospital) and Department of Pediatrics, Harvard Medical School, Boston, MA 02115, USA

INTRODUCTION

Recent advances on glycobiology research have rapidly expanded our knowledge of the importance of oligosaccharide structure in bacterial interaction with host cells. Cellsurface carbohydrates with a structural diversity are potential candidates as recognition sites for bacteria and their toxins. The recognition sites or "receptors" may exist on different glycoproteins and/or glycolipids. Several lines of evidence indicate that glycolipids are much more common than glycoproteins as receptors for bacterial adherence and toxin binding (*Karlsson*, 1989). Furthermore, cell-surface carbohydrates are expressed in a species-specific, tissue-specific and developmentally regulated manner (*Hakomori*, 1981; *Feize*, 1985, *Rademacher* et al., 1988). Therefore, the control mechanisms by which the expression of cell-surface carbohydrates are regulated may have a direct impact on the host, age and tissue specificities of different bacterial infections. In this article, we will summarise available information regarding intestinal receptor expression and its relevance to the host response to bacterial toxins.

MEMBRANE RECEPTORS FOR BACTERIAL TOXINS

Cholera and *Escherichia coli* enterotoxins

Table 1 summarises membrane receptors for several bacterial toxins that cause infectious diarrhoea. The best-known glycolipid receptor is the ganglioside GM1 for cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT) (*Eidels* et al., 1983). Both the terminal galactose and sialic acid of GM1 are required for the binding. These two toxins also bind to receptor variants, N-glycolyl-GM1 and GD1b, in human intestine, but with a much lower affinity (*Holmgren* et al., 1985). In addition, LT binds to a galactoprotein receptor in human (*Holmgren* et al., 1985), rabbit (*Holmgren* et al., 1982; *Griffiths* et al., 1986) and rat (*Zelmelman* et al., 1989) intestine. The receptor for the *E. coli* heat-stable toxin (STa) has been identified as a glycoprotein of 200 kilodaltons in rat intestine (*Kuno* et al., 1986). This STa receptor contains three peptides (80, 68 and 60 kilodaltons) with binding epitopes for the toxin, but the specific role of the sugar sequence in receptor binding bas not been established.

Clostridium difficile toxin

Toxin A from *Clostridium difficile* binds to hamster intestinal (*Krivan* et al., 1986) and rabbit erythrocyte (*Clark* et al., 1987) glycolipids with a terminal Galα1-3Galβ1-4GlcNAc sequence. The

Table 1: Membrane Receptors for Bacterial Toxins

Microbe	*Vibrio cholera*	
Toxin	Cholera toxin (84 KD, AB_5)	
Receptor	GM1	Galb1-3GalNAcb1-4 (NeuAca2-3)Galb1-4Glcb1-1Cer
Microbe	*Escherichia coli* (ETEC)	
Toxin	Heat-labile toxin (91 KD, AB_5)	
Receptor	GM1	Galb1-3GalNAcb1-4 (NeuAca2-3)Galb1-4Glcb1-1Cer
Gacactoprotein	(Determinant probably like GM1)	
Microbe	*Escherichia coli* (ETEC)	
Toxin	Heat-stable toxin STa (2 KD)	
Receptor	Glycoprotein (Determinant unknown)	
Microbe	*Clostridium difficile*	
Toxin	Toxin A (250 KD)	
Receptor	nLC_5Cer	Gala1-3Galb1-4 GacNAclb1-3Glcb1-4Glcb1-1Cer
Microbe	*Shigella dysenteriae*	
Toxin	Shiga toxin (70 KD, AB_5)	
Receptor	Gb3	Gala1-4Galb1-4Glcb1-1Cer
Microbe	*Escherichia coli* (EPEC and EHEC)	
Toxin	Shiga-like toxin (70 KD, AB_5)	
Receptor	Gb3	Gala1-4Galb1-4Glcb1-1Cer

proposed receptor sequence primarily exists as neolactopentaosylceramide (nLc_5Cer,Galα1-3Galβ1-4GlcNAcβ1-4Galβ1-4Glcβ1-1Cer) on the cell surface. However, the presence of this glycolipid structure in the human large intestine, the target site for toxin action, has yet to be detected (*Karlsson*, 1989).

Shiga and Shiga-like toxins

Shiga toxin is a protein cytotoxin produced by *Shigella dysenteriae* type 1. The glycolipid receptor for Shiga toxin has been identified as globotriaosylceramide (Gb3, Galα1-4Galβ1-4Glcβ1-1Cer) in rabbit intestine and HeLa cells (*Jacewicz* et al., 1986; *Lindberg* et al., 1987; *Mobassaleh* et al., 1988). *Escherichia coli* can produce cytotoxins that are similar in both structure and function to Shiga toxin. It has been shown that the Shiga-like toxin (or Verotoxin) also binds to the glycolipid Gb3 as a functional receptor (*Samuel* et al., 1990).

MEMBRANE RECEPTORS FOR BACTERIAL CELLS

Attachment to the host is required for the colonisation or infection with bacteria. This step is considered an important virulence factor in microbes. Cell-cell interaction between the host and bacteria is mediated by specific molecular recognition. The substances involved are bacterial proteins called adhesins and receptor proteins or carbohydrates on the epithelial surface. Adhesin molecules are expressed as bacterial cell surface components known as fimbriae or pili. The "receptor" sites are cell-surface glycoconjugates of the host. A bacterial cell may have a primary and secondary receptor

Table 2: Membrane attachment sites for bacterial cells

Sugar specificity or receptor epitope	Microbe	Adhesin
Mannose	*E. coli*	Type 1 fimbriae
Sialic acid		
NeuAcα2-8	*E. coli*	CFA/I
NeuAc, NeuGc	*E. coli*	CS2 or CFA/II
NeuGcα2-3Galβ1-4Glcβ1-1Cer	*E. coli*	K99 fimbriae
Galabiose		
Galα1-4Gal	*E. coli* (Uropathogenic)	Type P fimbriae
Lactosylceramide		
Galβ1-4Glcβ1-1Cer	*Bacteroides* *Lactobacillus* *Fusobacterium* *Clostridium* *Shigella* *Vibrio cholera*	

and the receptor epitope can be either a terminal or internal sugar sequence. Table 2 lists several species of bacteria that recognise specific sugar sequences on cell-surface glycoconjugates of the host.

Mannose receptor

Type 1 fimbriae are common on most *E. coli* bacteria and show mannose-sensitive haemagglutination (*Sharon*, 1987). Since glycolipids do not contain mannose, type 1 fimbriated *E. coli* binds to mannose receptors on glycoproteins instead.

Sialic acid receptor

Colonising factor antigens (CFA/I and CFA/II) of enterotoxigenic *E. coli* (ETEC) strains are known to be associated with infantile diarrhoea and travellers' diarrhoea in humans. CFA/I binds to sialic acid receptors with NeuAcα2-8 specificity (*Lindahl* et al., 1982). Recently, CS2 subtype of *E. coli* CFA/II has been shown to bind to a sialic acid (NeuAc or NeuGc)-containing receptor (*Sjoberg* et al., 1988). K99 is a colonising factor antigen of ETEC that causes enteric disease in piglets, calves and lambs. The glycolipid receptor for *E. coli* K99 has been identified as N-glycolyl-GM3 (NeuGcα2-3Galβ1-4Glcβ1-1Cer) (*One* et al., 1989; *Kyogashima* et al., 1989).

Galabiose receptor

Type P fimbriae of uropathogenic *E. coli* recognise the glycolipid receptor containing Galα1-4Gal (galabiose) sequence (*Bock* et al., 1985). This is the same receptor sequence recognised by Shiga toxin.

Lactosylceramide receptor

Many bacteria carry the lactosylceramide (Galβ1-4Glcβ1-1Cer) specificity with a relatively low binding affinity (*Karlsson*, 1989). Both Gram-negative and Gram-positive bacteria with various colonisation tissues are binders including both normal flora and pathogens. The dominate anaerobes of normal flora in the human

Table 3: developmental changes in the levels of toxin receptors in the small intestine

Toxin	Receptor	Species	Change with age
Cholera toxin GM1		rat	↔
E. coli LT	GM1	rat	↔
	Galactoprotein	rat	↑
E coli STa	Glycoprotein	rat	↓
		human	↓
Shiga toxin	Gb3	rabbit	↑
		rat	↑
C. difficile toxin A	nLc_5Cer	rabbit	↑

large intestine, including *Bacteroides*, *Clostridium*, *Fusobacterium* and *Lactobacillus*, may all use this specificity. *Vibrio cholerae* and *Shigella dysenteriae* are also shown to express lactosylceramide binding. These bacteria may compete for common receptor binding sites for adherence and colonisation. This competition may explain why overgrowth of *C. difficile* organism is induced after antibiotic treatment, which eliminates antibiotic sensitive strains such as *Bacteroides* (*van der Waaij*, 1989). Thus, competition for common attachment sites could be one of the important mechanisms by which normal flora of the gastrointestinal tract contribute to the protective barrier to intestinal colonisation by pathogens.

DEVELOPMENTAL CHANGES IN MEMBRANE RECEPTORS AND HOST RESPONSE

Increased toxigenic diarrhoea in the immature intestine due to receptor-dependent and -independent mechanisms

Age-related differences in the structure and function of membrane receptors for bacterial toxins (Table 3) have been studied using binding assays or glycolipid analysis. Table 4 summarises developmental variations in the host susceptibility to various bacteria-induced diarrhoeal diseases. In the case of glycolipid GM1, there is no change in the level of this glycolipid receptor in rat intestine during postnatal development (*Chu* et al., 1989). So the increased host responsiveness of the immature enterocyte to cholera toxin (*Chu* et al., 1989) and *E. coli* heatlabile toxin (LT, unpublished data) seems to be independent of the initial receptor occupancy (*Chu* et al., 1989). In contrast, the increased enterocyte response to *E. coli* heatstable toxin (STa) can be explained by an increased receptor number in the immature gut of rats (*Cohen* et al., 1986) and young children (*Cohen* et al., 1988). These two examples suggest that both receptor and/or postreceptor events may contribute to the developmental variations in host responsiveness to toxigenic diarrhoea. One of the postreceptor events appears to be due to the

Table 4: Age-related changes in the host susceptibility to infectious diarrhea in children and animal models

Pathogen	Host	Target tissue	Change with age
Vibro cholera	children	small intestine	↓
Enterotoxigenic *E. coli* (ETEC)	children	small intestine	↓
Clostridium difficile	children rabbit	large intestine ileum	↑ ↑
Shigella dysenteriae type 1	children rabbit	large intestine ileum	↑ ↑

underdeveloped activity of Na^+,K^+-ATPase (*Chu* et al., 1989), the sodium pump, that is the driving force for the intestinal absorption of ions and water. In addition, another functional galactoprotein receptor for LT is expressed in the adult, but not in the neonatal rat intestine (*Zelmelman* et al., 1989). Because of this glycoprotein's low binding affinity for LT, naturally occurring quantities of toxin most likely affect the epithelium primarily via the GM1 high affinity receptor in the rat intestine. However, the possibility that the LT glycoprotein receptor may play a role in travellers' diarrhoea in adult humans cannot be excluded.

Protection from toxigenic diarrhoea in the immature intestine due to a receptor-dependent decrease in host responsiveness

In the case of Shiga toxin and *C. difficile* toxin A, a positive correlation between the receptor expression and toxin effects has been demonstrated. Age-dependent increases in the expression of the glycolipid receptor Gb3 for Shiga toxin have been noted in the rabbit intestine (*Mobassaleh* et al, 1988; *Mobassaleh* et al., 1989). In rats, Gb3 is the major glycolipid of crypt cells of adult intestine and is detectable only after weaning (*Bonhours* and *Bonhours*, 1981). Similarly, the binding of *C. difficile* toxin A to membrane receptors in the rabbit intestine is shown to increase after weaning (*Eglow* et al., 1989). The underdeveloped expression of toxin receptors in the immature intestine could explain the relative resistance of human neonates to clinical shigellosis (*Keusch*, 1982). It could also explain the finding that infancy is the only population in which *C. difficile* toxin A is frequently detected in the absence of any clinical symptoms (*van der Waaij*, 1989).

MECHANISM FOR DEVELOPMENTAL CONTROL OF RECEPTOR EXPRESSION

Developmental regulation of glycosyltransferase and surface-carbohydrate expression

As mentioned, a defined oligosaccharide structure is needed for the binding of bacterial toxins and cells to membrane receptors. Therefore, the regulation of specific sugar sequence expression can control the expression of bacterial receptors during development. Although mechanisms for developmental regulated carbohydrate expression on the intestinal surface are not completely understood, one of the

Table 5: Developmental changes in the intestinal glycosyltransferase activities

Glycosyl-transferase	Donor substrate	Sequence formed	Change with age
Sialyltransferase (ST)			↓
Galα2,6-ST	CMP-NeuAc	**NeuAcα2-6**Galβ1-4GlcNAc-R	
Galα2,3-ST		**NeuAcα2-3**Galβ1-4GlcNAc-R	
Fucosyltransferase (FucT)			↑
GlcNAcα1,3-FucT	GDP-Fuc	Galβ1-4 (**Fucα1-3**)GlcNAc-R	
Galα1,2-FucT		**Fucα1-2**Galβ1-4GlcNAc-R	
		Fucα1-2 Galβ1-3GlcNAc-R	
Galactosyltransferase (GalT)			
GalNAcβ1,4-GalT	UDP-Gal	**Galβ1-4**GlcNAc-R	↑
Galα1,3-GalT		**Galβ1-3**Galβ1-4GlcNAc-R	↑
N-Acetylgalactosaminyl-transferase (GalNAcT)			
Galα1,3-GalNAcT	UDP-GalNAc	**GalNAcα1-3**(Fucα1-2)Gal-R	↑

major regulatory mechanisms appears to be operated via control of glycosyltransferase expres-sion. Table 5 illustrates examples of several glycosyltransferase activities of rat intestine that are under developmental control. A reciprocal relationship between a decrease in sialyltransferase activity and an increase in fucosyltransferase activity (*Chu* and *Walker*, 1986) appears to be well correlated with a shift from sialylation to fucosylation of microvillus membrane glycoproteins and glycolipids in the rat small intestine after weaning (*Torres-Pinedo* and *Mohmood*, 1984; *Pang* et al., 1987). Specifically, the increased sialylation in the neonatal intestine is reflected by an increased level of gangliosides (i.e. sialic acid containing glycolipids), mainly as the form of GM3 (*Bouhours* and *Bouhours*, 1983). Furthermore, Western blots of microvillus membrane glycoproteins probed with a sialic acid (α2-3)-specific lectin, MAA (*Maackia amurensis* agglutinin) and a sialic acid (α2-6)-specific lectin, SNA (*Sambucus nigra* agglutinin) also showed an increase in both α(2-3)- and α(2-6)-linked sialoglycoproteins in the immature intestine (unpublished data). It is our hypothesis that the presence of the sialic acid-rich glycoconjugates may possibly cause a barrier dysfucntion vis-à-vis receptor availability and binding capacity in the neonatal mucosa, and therefore may favour the colonisation and penetration of sialic acid-binding bacteria (e.g. ETEC) leading to neonatal bacterial infection. Two other glycosyltransferases, galactosyl- (*Pang* et al., 1987) and N-acetylgalactosylaminyltransferase activities (*Biol* et al., 1987), are noted to increase with age. The increased activities of these two enzymes may enhance the expression of glycolipids and glycoproteins containing galactose and N-acetylgalactosamine in the mature intestine, but their role in affecting the expression of sugar sequences involved in toxins receptors remains to be determined.

Regulation of glycolipid biosynthesis and toxin receptor expression

Most of the biosynthetic pathways for both acidic (i.e. gangliosides) and neutral glycolipids are well established

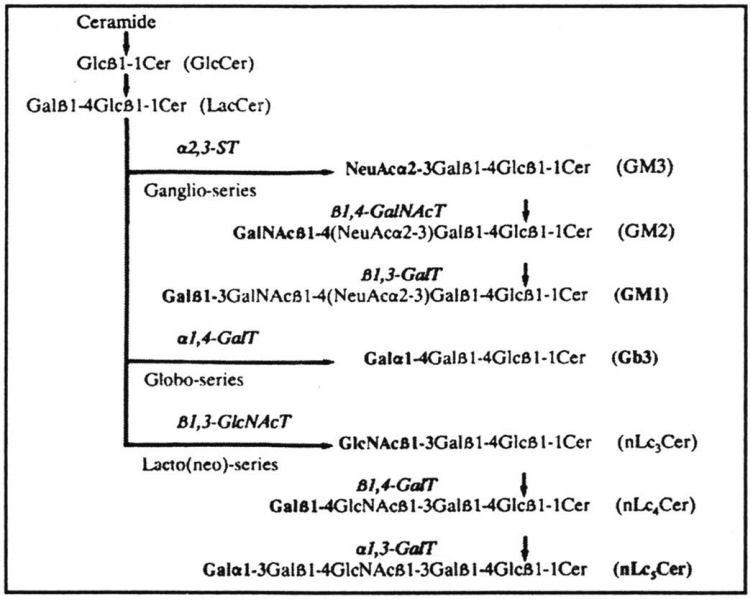


Figure 1: Biosynthetic pathways of glycolipid receptors for bacterial toxins.
Glycosyltransferases involved in these pathways are α2,3-sialyltransferase (α2,3-ST), β1,4-N-acetylgalactosaminyltransferase (β1,4-GalNAcT), β1,3-galactosyltransferase (β1,3-GalT) for cholera toxin receptor GM1; α1,4-galactosyltransferase (α1,4-GalT) for Shiga toxin receptor Gb3; and β1,3-N-acetylglycosyltransferase (β1,3-GlcNAcT), β1,4-galactosyltransferase (β1,4-GalT) and α1,3-galactosyltransferase (α1,3-GalT) for *C. difficile* toxin A receptor nLc_5Cer.

(*Wiegant*, 1985). Figure 1 depicts the biosynthetic pathways leading to the expression of glycolipid molecules, which are known receptors for bacterial toxins. Lactosylceramide (LacCer, Galβ1-4Glcβ1-1Cer) is the common precursor for the expression of the glycolipid receptors for cholera toxin, Shiga toxin, and *C. difficile* toxin A. Ganglioside GM1, the receptor for cholera toxin, is formed by the sequential addition of sialic acid, N-acetylgalactosamine and galactose to LacCer, catalysed by three enzymes, α2,3-sialyl-, β1,4-N-acetylgalactosaminyl-, and β1,3-galactosyltransferases, respectively. Shiga toxin receptor, Gb3, is formed by the addition of a galactose to LacCer, catalysed by α1,4-galactosyltransferase. *Clostridium difficile* toxin A receptor, neolactopentaosylceramide (nLc_5Cer) is formed by the sequential addition of N-acetylglucosamine, β-linked galactose and α-linked galactose, catalysed by β1,3-N-acetylglucosyl-, β1,4-galactosyl-, and α1,3-galactosyltransferases, respectively. Conceivably, developmental regulation of these lipid glycosyltransferases, especially the ones involved in the rate-limiting step of the glycosylation pathway, can markedly influence the expression and prevalence of toxin receptors on the intestinal surface during development. It may be assumed that the absence of receptor expression for both Shiga toxin and *C. difficile* toxin A is due to the underdevelopment of glycosyltransferases involved in the synthesis of Gb3 and of nLcCer as proposed in Figure 2. However, additional studies on developmental changes of these glycolipid glycosyltransferases in the gastrointestinal tract are needed to prove this hypothesis.

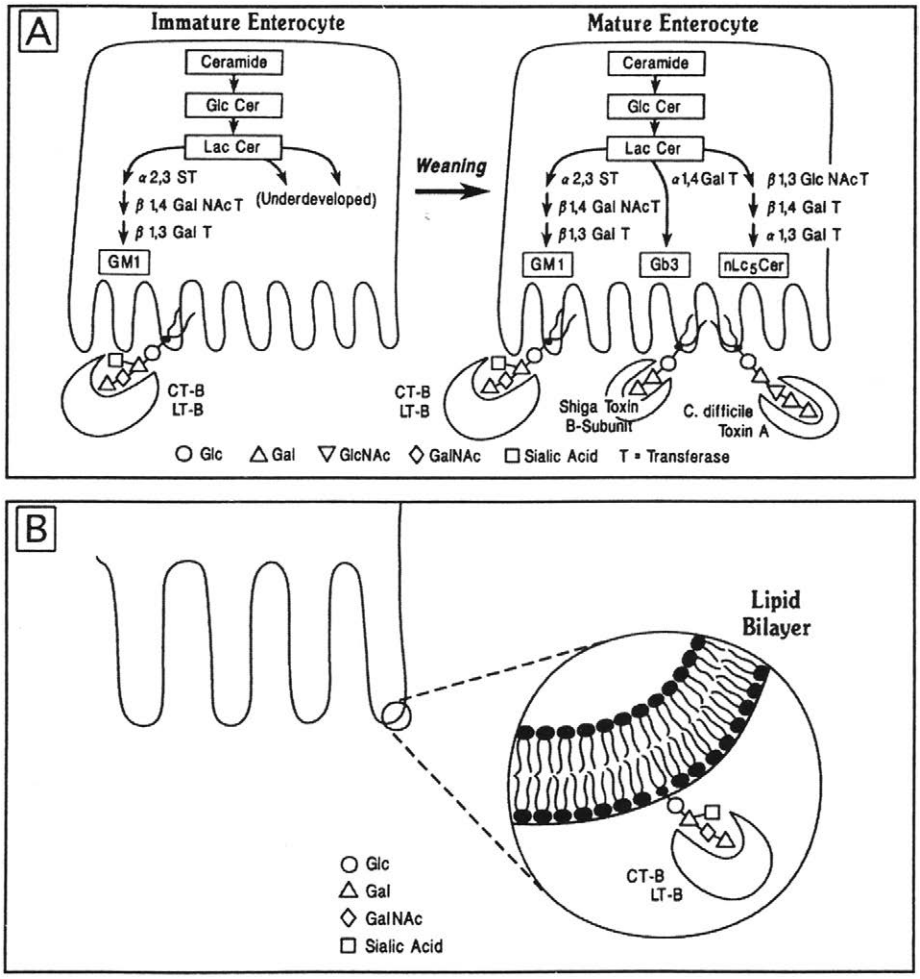


Figure 2: A proposed model for the control of glycolipid biosynthesis and toxin receptor expression during enterocyte development.
A. Immature vs. mature enterocytes
B. Detail of microvillus membrane showing expression of toxin receptor on lipid bilayer.

External factors that may influence developmental regulation

Alterations in the carbohydrate structures of glycolipids can be attributed to the variable level of glycosyltransferase activity during development. However, the glycoconjugate patterns formed through activity of glycosyltransferases in tissues may vary extensively depending on a number of external factors. These include the presence of chemical stimuli such as phorbol ester (*Ozaki* et al., 1989) and sodium butyrate (*Simmons* et al., 1975), or nutritional factors such as a vitamin A deficiency (*DeLuca* et al., 1970), dietary composition changes (*Biol* et al., 1984), or hormone stimuli such as cortisone (*Ozaki* et al., 1989; *Sato* et al., 1984) and thyroxin (*Sato* et al., 1984) administration, as well as the stimuli derived from bacterial colonisation itself (*Ozaki* et al., 1988; *Umesaki* et al., 1982; *Lucas* et al., 1989). These external factors may affect the developmental signals which modulate the

control of gene expression of glycosyltransferases probably at the transcriptional level (*Chu* et al., 1990; *Paulson* and *Colley*, 1989). On the other hand, other regulatory mechanisms may operate through the regulation of substrate availability as well as through the phosphorylation-dephosphorylation regulation (*Burczak* et al., 1984) of enzyme activities responsible for specific sugar sequences. Thus, intestinal microflora and nutritional conditions may modulate regulatory mediators that regulate the neonatal expression of bacterial receptors during development. However, these observations need more definitive studies to establish the specific mechanism of developmental regulation.

CONCLUSIONS AND SPECULATION

The molecular nature of membrane receptors for bacterial cells and toxins on intestinal epithelial cells points to the importance of the intestinal surface-carbohydrate expression in host-pathogen interactions. With the improved techniques for characterising receptor binding and receptor biochemical structure, the availability of several human intestinal epithelial cell lines (e.g. T84, HT-29, Caco-2) and of carbohydrate-specific monoclonal antibodies, we may identify additional membrane receptors and the receptor sugar sequences in the near future. Subsequent studies on the intestinal expression and developmental regulation of individual glycosyltransferases responsible for the addition of receptor sugar sequences should be pursued. By understanding the molecular nature of bacterial receptors in the intestine, developmental programming and environmental influence on receptor expression, and biological significance in neonatal host defences, new approaches may soon be available in the prevention and treatment of young infants with bacterial diseases.

ACKNOWLEDGEMENT

This work is supported in part by grants DK-37521, HD-12437 and DK-33506 from the National Institutes of Health.

LITERATURE

Biol, M.C., Martin, A., Paulin, C., Hemming, F., Louisot, P., and Richard, M.: Glycosyltransferase activities in the intestinal mucosa: Comparison between standard commercial and semi-synthetic diets. Ann. Nutr. Metab. 28, 52-64 (1984).

Biol, M.C., Richard, M.M., and Louisot, P.: Developmental changes in intestinal glycosyl-transferase activities. Pediatr. Res. 22, 250-257 (1987).

Bock, K., Breimer, M.E., Brignole, A., Hansson, G.C., and Karlsson, K.-A.: Specificity of binding of a strain of uropathogenic *Escherichia coli* to Galα1-4Gal-containing glycosphingolipids. J. Biol. Chem. 260, 8545-8551 (1985).

Bouhours, D. and Bouhours, J.F.: Developmental changes of rat intestinal glycolipids. Biochem. Biophys. Res. Commun. 99, 1384-1389 (1981).

Bouhours, D. and Bouhours, J. F.: Developmental changes of hematoside of rat small intestine: Postnatal hydroxylation of fatty acids and sialic acid. J. Biol. Chem. 258, 299-304 (1983).

Burczak, J.D., Soltysiak, R.M., and Sweeley, C.C.: Regulation of membrane-bound enzymes of glycosphingolipid biosynthesis. J. Lipid Res. 25, 1541-1547 (1984).

Chu, S.W. and Walker, W.A.: Developmental changes in the activities of sialyl- and fucosyl-transferases in rat small intestine. Biochim. Biophys. Acta 883, 496-500 (1986).

Chu, S.W., Ely, I.G., and Walker, W.A.: Age and cortisone alter host responsiveness to cholera toxin in the developing gut. Am. J. Physiol. 256 (Gastrointest. Liver Physiol. 19), G220-G225 (1989).

Chu, S.W., Zemelman, B.V., Ozaki, C.K., and Walker, W.A.: Evidence for region-specific regulation and cortisone induction of sialyltransferase mRNA expression in the developing intestine. J. Cell. Biochem. Suppl. 14B, 198 (1990).

Clark, G.F., Krivan, H.C., Wilins, T.D., and Smith, D.F.: Toxin A from *Clostridium difficile* binds to rabbit erythrocyte glycolipids with terminal Galα1-3Galβ1-4GlcNAc sequences. Arch. Biochem. Biophys. 257, 217-229 (1987).

Cohen, M.B., Moyer, M.S., Luttrell, M., and Giannella, R.A.: The immature rat small intestine exhibits an increased sensitivity and response to *Escherichia coli* heat-stable enterotoxin. Pediatr. Res. 20, 555-560 (1986).

Cohen, M.B., Guarino, B.A., Shukla, R., and Giannella, R.A.: Age-related differences in receptors for *Escherichia coli* heat-stable enterotoxin in the small and large intestine of children. Gastroenterology 94, 367-373 (1988).

DeLuca, L, Schumacher, M., and Wolf, G.: Biosynthesis of a fucose-containing peptide from small intestine in normal and vitamin A-deficient conditions. J. Biol. Chem. 245, 4551-4558 (1970).

Eglow, R., Pothoulakis, C., Israel, E., Walker, W.A., and LaMont, J.G.: Age-related increase in receptor binding for *Clostridium difficile* toxin A (TXA) on rabbit intestine. Gastroenterology 96, A136 (1989).

Eidels, L., Proia, R.L., and Hart, D.A.: Membrane receptors for bacterial toxins. Microbiol. Rev. 47, 596-620 (1983).

Feize, T.: Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens. Nature 314, 53-57 (1985).

Griffiths, S.L., Finkelstein, R.A., and Crithley, R.R.: Characterization of the receptor for cholera toxin and *Escherichia coli* heat-labile toxin in rabbit intestinal brush border. Biochem. J. 238, 313-322 (1986).

Hakomori, S.: Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. Annu. Rev. Biochem. 50, 733-764 (1981).

Holmgren, J., Fredman, P., Lindbald, M., Svennerholm, A.-M., and Svennerholm, L.: Rabbit intestinal glycoprotein receptor for *Escherichia coli* enterotoxin lacking affinity for cholera toxin. Infect. Immun. 38, 424-433 (1982).

Holmgren, J., Lindblad, M., Fredman, P., Svennerholm, L., and Myrvold, H.: Comparison of receptors for cholera and *Escherichia coli* enterotoxins in human intestine. Gastroenterology 89, 27-35 (1985).

Jacewicz, M., Clausen, M., Nudelman, E., Donohye-Rolfe, A., and Keusch, G.T.: Pathogenesis of *Shigella* diarrhea. XI. Isolation of a *Shigella* toxin binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide. J. Exp. Med. 163, 1391-1404 (1986).

Karlsson, K.-A.: Animal glycosphingolipids as membrane attachment sites for bacteria. Annu. Rev. Biochem. 58, 309-350 (1989).

Keusch, G.T.: Shigellosis. In: Bacterial infections of humans (Eds.: Evans, A.S. and Feldman, H.A.). Plenum Publishing Corporation, New York, NY, 487-509 (1982).

Krivan, H.C., Clark, G.F., Smith, D.F., and Wilkins, T.D.: Cell surface binding site for *Clostridium difficile* enterotoxin: Evidence

for a glycoconjugate containing the sequence Galα1-3Galβ1-4GlcNAc. Infect. Immun. 53, 573-581 (1986).

Kuno, K., Kamisaki, Y., Waldman, S.A., Gariepy, J., Schoolnik, G., and Murad, F.: Characterization of the receptor for heat-stable enterotoxins from *Escherichia coli* in rat intestine. J. Biol. Chem. 261, 1470-1476 (1986).

Kyogashima, M., Ginsburg, V., Krivan, H.: *Escherichia coli* K99 binds to N-glycolyl-sialoparagloboside and N-glycolyl-GM3 found in piglet small intestine. Arch. Biochem. Biophys. 270, 391-397 (1989).

Lindahl, M., Faris, A., and Wadstrom, T.: Colonization factor antigen on enterotoxigenic *Escherichia coli* is a sialic acid-specific lectin. Lancet ii, 280 (1982).

Lindberg, A.A., Brown, J.E., Stromberg, N., Westling-Ryd, M., Schulz, J.E., and Karlsson K.-A.: Identification of the carbohydrate receptor for Shiga toxin produced by *Shigella dysenteriae*, type I. J. Biol. Chem. 262, 1779-1785 (1987).

Lucas, F., Elmer, G.W., Brot-Laroche, E., and Corthier, G.: Fixation of *Clostridium difficile* toxin A and cholera toxin to intestinal brush border membranes from axenic and conventional mice. Infect. Immun. 57, 1680-1683 (1989).

Mobassaleh, M., Donohue-Rolfe, A., Jacewicz, M., Keusch, G., and Grand, R.: Pathogenesis of *Shigella* diarrhea. XIII. Evidence for a developmentally regulated glycolipid receptor for *Shigella* toxin involved in the fluid secretory response of rabbit small intestine. J. Infect. Dis. 157, 1023-1031 (1988).

Mobassaleh, M., Gross, S.K., McCluer, R.H., Donohue-Rolfe, A., and Keusch, G.T.: Quantitation of the rabbit intestinal glycolipid receptor for Shiga toxin: Further evidence for the developmental regulation of globotriaosylceramide in microvillus membrane. Gastroenterology 97, 384-391 (1989).

One, E., Abe, K., Nakazawa, M., and Naiki, M.: Ganglioside epitope recognized by K99 fimbriae from enterotoxigenic *Escherichia coli*. Infect. Immun. 57, 907-911 (1989).

Ozaki, C.K., Chu, S.W., Ely, I.G., and Walker, W.A.: Effect of a germ-free environment on the development of intestinal glycosyltransferases in rats. FASEB J. 2, A650 (1988).

Ozaki, C.K., Chu, S.W., and Walker, W.A.: Developmental changes in galactosyltransferase activity in the rat small intestine. Biochim. Biophys. Acta 991, 243-247 (1989).

Pang, K.Y., Bresson, J.L., and Walker, W.A.: Development of gastrointestinal surface. VIII. Lectin identification of carbohydrate differences. Am. J. Physiol. 252 (Gastrointest. Liver Physiol 15), G685-G691 (1987).

Paulson, J.C., and Colley, K.J.: Glycosyltransferases: Structure, localization, and control of cell type-specific glycosylation. J. Biol. Chem. 264, 17615-17618 (1989).

Rademacher, T.W., Parekh, R.B., and Dwek, R.A.: Glycobiology. Annu. Rev. Biochem. 57, 785-838 (1988).

Samuel, J.E., Perera, L.P., Ward, S., O'brien, A.D., Ginsburg, V., and Krivan, H.C.: Comparison of the glycolipid receptor specificities of Shiga-like toxin type II and Shiga-like toxin type II variants. Infect. Immun. 58, 611-618 (1990).

Sato, E., Fujie, M., Uezato, T., Fujita, M., and Nishimura, K.: Hormonal effects on the developmental changes of mouse small intestinal glycolipids. Biochem. Biophys. Res. Commun. 119, 1168-1173 (1984)

Sharon, N.: Bacterial lectins, cell-cell recognition and infectious disease. FEBS Lett. 217, 145-157 (1987).

Simmons, J.F., Fishman, P.R., Freese, L, and Brady, R.O.: Morphological alterations and ganglioside sialyltransferase activity induced by small fatty acids in HeLa cells. J. Cell Biol. 66, 414-425 (1975).

Sjoberg, P.-O., Lindahl, M., Porath, J., and Wadstrom, T.: Purification and characterization of CS2, a sialic acid-specific haemagglutinin of enterotoxigenic *Escherichia coli*. Biochem. J. 255, 105-111 (1988).

Torres-Pinedo, R. and Mohmood, A.: Postnatal

changes in biosynthesis of microvillus membrane glycans of rat small intestine. I. Evidence of a developmental shift from terminal sialylation to fucosylation. Biochem. Biophys. Res. commun. 125, 546-553 (1984).

Umesaki, Y., Sakata, T., and Yajuma, T.: Abrupt induction of GDP-fucose:asialo GM1 fucosyltransferase in the small intestine after conventionalization of germ-free mice. Biochem. Biophys. Res. Commun. 105, 439-443 (1982).

van der Waaij, D.: The ecology of the human intestine and its consequences for overgrowth by pathogens such as *Clostridium difficile*. Annu. Rev. Microbiol. 43, 69-87 (1989).

Wiegandt, H.: Glycolipids. Elsevier Science Publishers, Amsterdam, (1985).

Zelmelman, B.V., Chu, S.W., and Walker, W.A.: Host response to *Escherichia coli* heat-labile exterotoxin via two microvillus membrane receptors in the rat intestine. Infect. Immun. 57, 2947-2952 (1989).

Old Herborn University Seminar Monograph 4:
Host-microflora interactions in the first years after birth.
Editors: Peter J. Heidt, Rial D. Rolfe, Volker Rusch, and Dirk van der Waaij.
Institute for Microbiology and Biochemistry, Herborn-Dill, Germany: 73-86 (2002).

PROMOTION OR INHIBITION OF BACTERIAL TRANSLOCATION FROM THE GI TRACT BY BACTERIAL COMPONENTS

RODNEY D. BERG

Department of Microbiology and Immunology,
Louisiana State University Medical Center, Shreveport, LA 71130, USA

SUMMARY

Bacterial translocation is defined as the passage of viable bacteria from the gastrointestinal (GI) tract across the mucosal epithelium to extra-intestinal sites. Bacterial translocation does not normally occur in the healthy adult animal due to the host immune defences, the physical barrier of an intact mucosa, and the indigenous microflora maintaining an ecological balance in the GI tract. Disruption of any of these defence mechanisms allows indigenous bacteria to translocate from the GI tract to the mesenteric lymph nodes (MLN) and other organs. The disruption of more than one mechanism allows the translocating bacteria to spread systemically to cause lethal sepsis. Various bacterial components can either promote or inhibit bacterial translocation. Bacterial endotoxin injected once intraperitoneally (i.p.) promotes the translocation of certain indigenous bacteria from the GI tract to the MLN. Histologic examination of the intestinal mucosa from these endotoxin-challenged mice reveals physical disruption of the mucosal barrier. Oral gavage prior to endotoxin injection with allopurinol, a competitive inhibitor of xanthine oxidase activity, decreases endotoxin-induced mucosal injury and subsequent bacterial translocation. Inactivation of xanthine oxidase activity due to placing mice on a molybdenum-free, tungstate diet for 14 days prior to endotoxin challenge also reduces mucosal injury and bacterial translocation. Thus, it appears that a primary mechanism promoting bacterial translocation from the GI tract after endotoxin challenge is mucosal injury caused by xanthine oxidase-generated oxidants.

Other bacterial components can inhibit bacterial translocation from the GI tract. One intraperitoneal injection of formalin-killed *Propionibacterium acnes*, a non-specific immunomodulator, inhibits the translocation of various Gram-negative, enteric bacilli, such as *E. coli*, from the GI tract to the MLN. Interestingly, *P. acnes* vaccination does not inhibit *E. coli* translocation in gnotobiotic mice monoassociated with *E. coli. P. acnes* vaccination inhibits *E. coli* translocation in 8-week-old gnotobiotic mice if they are colonised with the entire GI microflora within 1 week after birth, but not if they are colonised with the microflora beginning at 2 or 3 weeks after birth. Thus, it appears that antigenic stimulation by the indigenous GI microflora is required to prime the host immune response so that a subsequent *P. acnes* vaccination can activate macrophages to inhibit bacterial translocation. These experimental models utilising various bacterial components have proved useful in attempts to determine the pathophysiology of bacterial translocation and to delineate immune defence mechanisms.

Table 1: Mechanisms promoting bacterial translocation in various animal models

Animal model	Intestinal bacterial Overgrowth	Gut mucosal injury	Immunocompromised host defences
Oral antibiotics	+	-	-
Intestinal obstruction	+	+	?
Endotoxin	-	+	+
Thermal injury	-	±	+
Haemorrhagic shock	-		?
Diabetes	±	±	+
Leukaemia	-	-	+
Protein malnutrition	±	±	±

INTRODUCTION

Indigenous bacteria normally remain confined to the gastrointestinal (GI) tract in healthy, adult mice. However, under certain circumstances these indigenous bacteria cross the gut mucosal barrier to appear in extraintestinal sites, such as the mesenteric lymph node complex (MLN), spleen, liver, kidney, and blood. We have termed this passage of indigenous bacteria from the GI tract to other sites as bacterial translocation (*Berg* and *Garlington*, 1979). *Keller* and *Engley* (1958) appear to be the first to have used the term translocation when they described the passage of bacteriophage particles across the intestinal epithelium. Bacterial translocation was subsequently used to describe the passage of bacteria from the GI tract to the MLN in rats (*Wolochow* et al., 1966) and to the liver in chickens (*Fuller* and *Jayne-Williams*, 1970).

Bacterial translocation occurs when: (a) the host immune defences are compromised (*Berg*, 1983a; *Owens* and *Berg*, 1980; 1983), (b) the mucosal barrier is physically disrupted (*Morehouse* et al., 1986; *Deitch* et al., 1989a), or (c) there is intestinal bacterial overgrowth due to alterations in the ecologic equilibrium of the indigenous microflora (*Berg* and *Owens*, 1979; *Berg*, 1980a,b,c; *Steffen* and *Berg*, 1983). We have demonstrated bacterial translocation in a variety of animal models including rodents subjected to streptozotocin-induced diabetes (*Berg*, 1985), thermal injury (*Maejima* et al., 1984a,b), leukaemia (*Penn* et al., 1986), endotoxaemia (*Deitch* and *Berg*, 1987; *Deitch* et al., 1987a), haemorrhagic shock (*Baker* et al., 1987), malnutrition (*Deitch* et al., 1987b), intestinal obstruction (*Deitch* et al., 1990a) or bile ligation (*Deitch*, et al., 1990b). The mechanisms responsible for promoting bacterial translocation in these animal models are presented in Table 1 (*Berg*, 1980b, 1981b, 1983b, 1985). In some models, the host exhibits multiple deficiencies in defence mechanisms resulting in bacteraemia and lethal sepsis by translocating indigenous bacteria. Thus, the translocation of indigenous bacteria is a clinically important event even though the indigenous bacteria are relatively nonpathogenic, i.e. opportunistic pathogens.

Not all species of indigenous bacteria translocate from the GI tract with equal efficiency. The bacterial species found to translocate most readily from the GI tract in rodent models, namely the Gram-negative, facultatively anaerobic, enteric bacilli, such as *Escherichia coli, Proteus mirabilis,*

Klebsiella pneumoniae, Enterobacter cloacae, and aerobic *Pseudomonas aeruginosa* (*Steffen* et al., 1988), also are the bacterial species recognised as causing a large proportion of septicaemia in hospitalised patients. Interestingly, the strictly anaerobic bacteria, such as *Bacteroides fragilis*, *Clostridium*, and *Eubacterium*, translocate at the lowest rate of any of the bacteria tested to date even though they colonise the GI tract at extremely high levels (10^{10-11}/g caecum).

PROMOTION OF BACTERIAL TRANSLOCATION BY ENDOTOXIN

Large amounts of endotoxin, the lipopolysaccharide component of Gram-negative bacteria, are normally present in the GI tract since the GI tract harbours tremendous populations of Gram-negative, indigenous bacteria. Endotoxin is released during bacterial cell growth and particularly upon bacterial cell death and lysis. The lethal dose of endotoxin varies from species to species, ranging from 10 μg/kg body weight in the goat to 100 μg/kg in the rat. The lethal dose for humans is not known. However, the human is much more sensitive to the effects of endotoxin than are most other animals; 10^{-12} g/kg endotoxin produces symptoms in humans.

Only small amounts of endotoxin are absorbed from the healthy GI tract. Two mechanisms have been proposed to account for this poor endotoxin absorption: (a) the intestinal mucosa is relatively impermeable to endotoxin absorption and (b) bile salts bind directly to endotoxin in the GI lumen to form detergent-like complexes that are poorly absorbed (*Cahill* et al., 1987). The very small amounts of endotoxin that are absorbed from the healthy GI tract are detoxified by Kuppfer cells in the liver (*Nolan*, 1981). However, in severely ill or injured patients, bacteria and endotoxin can cross the gut mucosal barrier and gain access to tissues and the systemic circulation. Thus, endotoxin absorption from the GI tract is promoted by conditions that increase mucosal permeability or decrease bile output.

A relationship among shock, intestinal ischaemia, and endotoxaemia was first proposed by *Ravin* and *Fine* (1962). More recently, it is hypothesised that gut barrier failure in conjunction with heptatic dysfunction promote or potentiate the newly recognised multiple organ failure syndrome (MOF) (*Carrico* et al., 1986). MOF is a common final pathway leading to death in a variety of patients. Conditions such as shock, infection or immunosuppression increase gut mucosal permeability resulting in increased translocation of bacteria and bacterial products (e.g. endotoxin) from the GI tract to the portal and systemic circulations. Translocated endotoxin then activates various plasma protein cascades, resident macrophages, and circulating neutrophils releasing monokines and proteins that in turn further increase gut mucosal permeability.

Endotoxin is known to increase gut mucosal permeability and to decrease host immune defences. Since endotoxaemia is common in a variety of patients, we performed studies in rodent models to determine whether endotoxin also could promote the translocation of indigenous bacteria from the GI tract. Specific pathogen-free (SPF) mice were injected once intraperitoneally (i.p.), with *E. coli* O26:B6 endotoxin and the peritoneum, MLN, spleen, liver, and blood tested 24 hr later for

translocating indigenous bacteria (*Deitch* et al., 1987a). Endotoxin promoted bacterial translocation to the MLN in a dose-dependent fashion; 88% of the MLN were positive after i.p. injection with 2 mg endotoxin. The spleen and liver cultures were negative at all endotoxin doses. Thus, one i.p. injection of endotoxin promotes bacterial translocation from the GI tract to the MLN. However, the translocating bacteria remain confined to the MLN and do not spread to other organs, such as the spleen or liver.

Polymyxin B interferes with the biologic action of endotoxin by stoichiometrically binding to the endotoxin molecule (*Jacobs* and *Morrison*, 1977). Consequently, to demonstrate the specificity of endotoxin-induced bacterial translocation, mice were injected with *E. coli* O26:B6 or *E. coli* O111:B4 endotoxin that had been incubated with polymyxin B for 3 hr at 20°C and compared to other mice injected with endotoxin not reacted with polymyxin B. Mice injected with *E. coli* O26:B6 endotoxin exhibited 71% incidence of bacterial translocation to the MLN whereas mice injected with the polymyxin B-*E. coli* O26:B6 endotoxin mixture exhibited only a 29% translocation incidence (*Deitch* et al., 1989a). Mice injected with *E. coli* O111:B4 had 75% positive MLN cultures compared to 13% positive cultures in mice injected with the polymyxin B-*E. coli* O111:B4 endotoxin mixture. Gentamicin exhibits a similar bactericidal spectrum as polymyxin B but does not bind to endotoxin. Incubation of *E. coli* O111:B4 endotoxin with gentamicin rather than polymyxin B prior to injection did not decrease endotoxin-induced translocation. These results further confirm the specificity of endotoxin-induced bacterial translocation.

Interestingly, the caecal populations of Gram-negative, enteric bacilli, such as *E. coli*, increased 100-fold 24 hr following one i.p. endotoxin injection (*Deitch* et al., 1987a). By 48 hr following endotoxin injection, the caecal population levels of these bacteria returned to normal and concomitantly both the incidence and levels of bacterial translocation to the MLN also decreased. The mechanisms whereby endotoxin injection influences caecal population levels of indigenous bacteria are unknown, although endotoxin is known to reduce GI motility and to cause transient intestinal ischaemia. Zymosan, a yeast cell wall product unrelated to endotoxin that is inflammatory due to its activation of complement, also increased caecal population levels of indigenous enteric bacilli and promoted bacterial translocation to the MLN when injected i.p. (*Deitch* et al., 1992).

Endotoxin is composed of a core polysaccharide, a lipid-A component containing long-chain fatty acids linked to a glucosamine backbone, and polysaccharide side chains (the O-antigens). To determine which components of the endotoxin structure are important in promoting bacterial translocation, mice were injected with endotoxin from six R-mutant strains of *Salmonella* (Ra, Rb, Rc, Rd, Re, or lipid A). These R-mutants differ in their endotoxin compositions. Intact *Salmonella* endotoxin (wild type) and the Ra and Rb endotoxin fragments promoted bacterial translocation to the MLN whereas the *Salmonella* endotoxin fragments lacking the terminal-3 sugars of the core polysaccharide (Rc, Rd, Re, or lipid A) did not promote bacterial translocation (*Deitch* et al., 1989b). Thus, the terminal portion of the core polysaccharide of *Salmonella* endotoxin appears to be required to promote bacterial translocation. Only the endotoxin fragments that promoted bacterial translocation also

were associated with increased caecal population levels of Gram-negative, facultatively anaerobic, enteric bacilli. Also, injection of the translocation-promoting Ra fragment also increased ileal xanthine oxidase and xanthine dehydrogenase activities indicating mucosal injury, whereas the non-promoting Rc and Re fragments did not increase these enzymatic activities. Both lipid A and endotoxin appear to produce toxic manifestations by stimulating host cells, especially macrophages, to release mediator substances that then act as second messengers to disrupt various homeostatic systems. The finding that *Salmonella* lipid A and the Rc, Rd, or Re endotoxin fragments do not promote bacterial translocation suggests that endotoxin does not induce bacterial translocation primarily by inducing the liberation of macrophage products. This conclusion also is supported by our previous studies demonstrating that endotoxin injected i.p. also promotes bacterial translocation in C3H/HeJ mice that are genetically resistant to macrophage activation by endotoxin (*Deitch* et al., 1987a).

Endotoxin-sensitive C3HeB/FeJ mice and endotoxin-resistant C3H/HeJ mice were tested for bacterial translocation after endotoxin challenge to determine whether genetic sensitivity or resistance to endotoxin would be a factor in endotoxin-induced translocation (*Deitch* et al., 1987a). C3H/HeJ mice are known to exhibit a reduced response to endotoxin and, therefore, have been labelled endotoxin-resistant or hyporesponsive whereas C3HeB/FeJ mice exhibit an exaggerated physiologic or hyper-responsiveness to endotoxin. Endotoxin-sensitive C3HeB/FeJ mice did not exhibit spontaneous bacterial translocation nor did bacterial translocation occur after they received a low dose challenge with endotoxin (0.1 mg/kg i.p.) suggesting that this genetic sensitivity to endotoxin is not normally associated with spontaneous bacterial translocation. Also, translocation occurred at the same rate to the MLN in both endotoxin-resistant C3H/HeJ mice and outbred Ha/ICr CD-1 mice injected i.p. with 2 mg of endotoxin. Consequently, neither genetic sensitivity nor genetic resistance to endotoxin affected endotoxin-induced bacterial translocation suggesting that endotoxin does not promote translocation primarily by depressing or activating the immune system or by triggering increased activity of the lymphoproliferative system.

Histological examination of the GI tissue from endotoxin challenged mice revealed physical disruption of the mucosal barrier. The ileal and caecal lamina propria were oedematous and there was separation of the epithelium from the lamina propria in certain areas, such as at the tips of villi. The duodenal, jejunal, and colonic mucosa appeared normal. Thus, endotoxin-induced bacterial translocation appears to be due primarily to damage to the gut mucosal barrier.

Studies have implicated xanthine oxidase-generated, oxygen-free radicals as mediators of intestinal injury (*Parks* et al., 1982a). Consequently, we determined whether inhibition of xanthine oxidase activities by allopurinol or inactivation of xanthine oxidase by a sodium tungstate diet would prevent the mucosal damage and subsequent bacterial translocation occurring after endotoxin challenge. SPF CD-1 mice were antibiotic decontaminated for 4 days, monoassociated with streptomycin-resistant *E. coli* C25 and challenged once i.p. with *E. coli* O26:B6 endotoxin. The MLN were tested for translocating *E. coli* C25 24 hr later. One group of mice was given allopurinol by gastric lavage (50 mg/kg) 48 and 24 hr prior to i.p. endotoxin

Table 2: Effect of inhibition of inactivation of xanthine oxidase activity on *E. coli* O26:B8 endotoxin-induced bacterial translocation

Group	Translocation Incidence to MLN	CFU/MLN
Control	0%	0
Allopurinol	0%	0
Tungsten diet	0%	0
Endotoxin	80%	1,607
Allopurinol plus endotoxin	31%*	220**
Tungsten diet plus endotoxin	17%*	144**

* $p<0.01$
** $p<0.05$

challenge. Another group of mice was placed on a diet supplemented with sodium tungstate (0.7 g/kg diet), but restricted in molybdenum (ICN Bichemical, Cleveland, OH). This diet contains normal levels of protein, vitamins, minerals, and other trace elements. To deplete intestinal levels of xanthine oxidase, the mice were main-tained on this diet a minimum of 14 days prior to endotoxin challenge. Intestinal xanthine oxidase and xanthine dehydrogenase activities of the control, endotoxin-challenged, and tungstate diet-treated mice were determined by the method of *Ward* and *Rajagopolan* (1976) measuring xanthine oxidase-dependent uric acid formation. Both the incidence and level of bacterial translocation to the MLN after endotoxin challenge were significantly reduced by pre-treatment with allopurinol or the tungstate diet (Table 2). Furthermore, there was no significant intestinal mucosal damage in endotoxin-challenged mice pre-treated with allopurinol or the tungstate diet. Ileal xanthine oxidase activity also was reduced in the mice fed the tungstate diet.

To further strengthen these findings, the effect of allopurinol pre-treatment on bacterial translocation was tested in a bacterial overgrowth model in which translocation is promoted by an increase in caecal population levels rather than by damage to the gut mucosal barrier as in endotoxin-induced translocation. Allopurinol pre-treatment should not decrease bacterial translocation in this overgrowth model. SPF mice were antibiotic decontaminated, monoassociated with *E. coli* C25, and tested 4 days later for *E. coli* C25 translocation to the MLN. There was no difference in the rate of *E. coli* C25 translocation between the group of mice pre-treated with allopurinol and the non-treated control group suggesting that the reduction of endotoxin-induced bacterial translocation by allopurinol in the earlier experiments described above is a specific response related to the ability of allopurinol to inhibit xanthine oxidase activity and thereby reduce mucosal damage (*Deitch* et al., 1989b).

It was also determined whether selective inhibitors of platelet-activating factor (PAF) could reduce endotoxin-induced bacterial translocation (*Deitch* et al., 1989). PAF is produced by many cells, such as macrophages, neutrophils, platelets, and endothelial cells. The biologic effects of PAF include increased vascular permeability, hypotension, and death (*Braquet* et al., 1987). PAF as been implicated as the mediator of bowel necrosis induced by endotoxin or tumour necrosis factor (TNF) (*Sun* et al., 1988). PAF antago-

nist, SRI 63-441 or BN 52021 (20 mg/kg), was administered i.p. 30 min prior to challenge with either *E. coli* O111:B4 or *E. coli* O26:B6 endotoxin. Neither endotoxin-induced mucosal damage nor endotoxin-induced bacterial translocation were blocked by the PAF antagonists (*Deitch* et al., 1989b). Thus, endotoxin challenge does not appear to promote bacterial translocation from the GI tract by inducing the synthesis and/or release of various pro-inflammatory mediators.

One i.p. injection of endotoxin promotes bacterial translocation to the MLN but the translocating bacteria remain confined to the MLN and do not spread systemically to other organs. This same dose of endotoxin given i.p. to thermally-injured mice (25% total body surface area burn) promotes bacterial translocation to the MLN, spleen, liver and blood, and the mice die of sepsis caused by their indigenous GI bacteria (*Deitch* and *Berg*, 1987). Furthermore, the mortality rate of mice receiving only endotoxin or only thermal injury is less than 10% whereas the mortality rate is 100% in mice receiving the combination of endotoxin plus thermal injury. Protein malnourished mice (21 days on a low-protein whey diet; Tekland Test Diets, Madison, WI) also are more susceptible to endotoxin-induced bacterial translocation than are normally nourished mice (*Li* et al., 1989).

More study is required to define the relationships among endotoxaemia, xanthine oxidase activities, gut mucosal injury, bacterial translocation. and sepsis. Since endotoxin challenge also can reduce intestinal blood flow (*Morrison* and *Ryan*, 1987), it is possible that intestinal ischaemia induced by endotoxin could initiate the activation of xanthine oxidase (*Smith* et al., 1987). It also has been postulated that endotoxin increases intestinal permeability by the local action of vasoactive mediators acting within the gut wall (*Cuevas* and *Fine*, 1973). Another possibility is that endotoxin indirectly induces activation of xanthine oxidase through the liberation of secondary mediators, such as interferon.

INHIBITION OF BACTERIAL TRANSLOCATION BY NON-SPECIFIC MACROPHAGE STIMULATION

The host immune system is important in preventing bacterial translocation from the GI tract. It seems probable that the various compartments of the host immune system, such as secretory immunity, cell-mediated immunity, and serum immunity, all may be important to various degrees in protecting the host against bacterial translocation. Secretory IgA possibly could inhibit the close association or adherence of bacteria with the gut mucosa that must occur prior to their translocation across the mucosal barrier. However, the role of secretory IgA in preventing bacterial translocation has not been tested to date.

There is some evidence that T-cell mediated immunity is important in the immune defence against bacterial translocation. Spontaneous bacterial translocation from the GI tract to the MLN, spleen, liver and kidney readily occurs in genetically athymic (nu/nu) mice (*Owens* and *Berg*, 1980). Spontaneous bacterial translocation, however, is inhibited in adult nu/nu mice that have been grafted with thymuses when neonates (*Owens* and *Berg*, 1980). Bacterial translocation from the

Table 3: Inhibition of *E. coli* C25 translocation by *P. acnes* vaccination in antibiotic decontaminated mice monoassociated with *E. coli* C25

Mice	Spleen weight	CFU/g caecum	Translocation incidence	CFU/g MLN
Control	0.10	1.7×10^9	75% (23/29)	1,860
P. acnes	0.57	2.2×10^9	41%	304*

*$p<0.01$

GI tract also occurs in neonatally thymectomised mice (*Owens* and *Berg*, 1982). However, even though T-cell mediated immunity has been demonstrated to inhibit bacterial translocation, the mechanisms whereby this occurs have not been elucidated.

Serum immunoglobulins also are likely important in clearing translocating bacteria once they have entered the lamina propria, lymph, blood or reticulo-endothelial organs, such as the MLN. Certain indigenous bacteria readily translocate from the GI tract to the MLN, spleen, liver, and kidney. Mice injected intraperitoneally with immunosuppressive agents, such as prednisolone or cyclophosphamide, exhibited increased bacterial translocation to the MLN, spleen, liver, and kidney (*Berg*, 1983a). These mice also exhibited reduced spleen plaque-forming (PFC) responses to antigens of *E. coli.*

Resident macrophages in the MLN are ideally situated to clear translocating bacteria, since in most animal models demonstrating bacterial translocation, the translocating bacteria first appear in the MLN prior to their appearance in other organs, such as the liver or spleen (*Berg*, 1983b). Non-specific immunomodulators are available that non-specifically enhance the abilities of macrophages to engulf and kill bacteria. Since it cannot be predicted which of several species of bacteria will translocate in any particular clinical condition, it would be useful to develop an immunologic regimen that would inhibit the translocation of a broad range of bacterial species.

Consequently, we examined the effectiveness in preventing bacterial translocation of three such non-specific immunomodulators, namely glucan, muramyl dipeptide and killed *Propionibacterium acnes* (formerly classified as *Corynebacterium parvum*). Glucan is a polyglycan derived from the cell wall of *Saccharomyces cerevisiae* (*Hassid* et al., 1941) and increases both cell-mediated and humoral immunity (*Wooles* and *DiLuzio*, 1962). Muramyl dipeptide is a small molecular weight glycopeptide responsible for the adjuvant action of *Mycobacterium*. *P. acnes* is known to exert a multitude of effects on the immune system, the most important being the activation of macrophages. Formalin-killed *P. acnes* when injected into mice increases their resistance to a variety of pathogenic bacteria, such as *Salmonella* (*Collins* and *Scott*, 1974), *Listeria* (*Miyata* et al., 1980) and *Staphylococcus* (*Stinnett* et al., 1979).

SPF mice were antibiotic decontaminated with oral streptomycin (1 mg/ml) and penicillin G (100 U/ml) in their drinking water for 7 days. One group of mice then was injected once i.p. with 0.2 ml (1.4 mg) of formalin-killed *P. acnes* (Burroughs-Wellcome, Research Triangle, NC). Another group of mice was injected i.p. with 0.2 ml (0.4 mg) of particulate glucan-P (Accurate Chemical and Scientific Corp., Westbury, NY) on days 10 and 12 following antibiotic decontamination. A

Table 4: Lack of inhibition of *P. acnes* vaccination on the translocation of *E. coli* C25 in gnotobiotic mice monoassociated with *E. coli* C25

Mice	Spleen weight	CFU/g caecum	Translocation incidence	CFU/g MLN
Controls	0.10	4.6×10^8	100% (20/20)	2,650
P. acnes	0.71	5.4×10^8	100% (20/20)	1,730

third group of mice was injected i.p. with muramyl dipeptide. On day 10, the mice were challenged by giving *E. coli* C25 (1×10^9/ml) in their drinking water. The mice were sacrificed on day 14 and the MLN cultured for translocating E. *coli* C25. The population levels of *E. coli* C25 in the caecum also were determined. Vaccination with killed *P. acnes* reduced both the incidence and numbers of *E. coli* C25 translocating to the MLN when compared with control mice injected with saline (Table 3). Neither glucan nor muramyl dipetide inhibited *E. coli* C25 translocation to the MLN. The caecal levels of *E. coli* C25 were similar in all groups of mice. Splenomegaly, as an indicator of the stimulatory effect of the immunomodulator, was more pronounced after injection with *P. acnes* than after injection with glucan. In other experiments, vaccination with *P. acnes* also decreased the translocation of *P. mirabilis* and *E. cloacae* to the MLN. Since killed *P. acnes* vaccination is reported to non-specifically activate macrophages (*Herbert* et al., 1983), it appears that macrophages are important effector cells in the host defence against translocation by Gram-negative, enteric bacilli.

The effectiveness of *P. acnes* vaccination in decreasing *E. coli* C25 translocation also was tested in monoassociated gnotobiotic mice. Germfree mice were injected intraperitoneally with 1.4 mg of formalin-killed *P. acnes* and monoassociated with *E. coli* C25 3 days later by placing an overnight culture in their drinking water (1×10^9/ml). On day 7, the mice were sacrificed and the MLN cultured for translocating *E. coli* C25 as described previously (*Berg* and *Garlington*, 1979). The splenic index of 7.1 (spleen weight of experimentals/spleen weight of controls) demonstrated that the monoassociated gnotobiotes were immunologically stimulated by the *P. acnes* vaccination (Table 4). However, there was not a decrease in the incidence or the numbers of *E. coli* C25 translocating to the MLN as compared with control, non-vaccinated gnotobiotes monoassociated with *E. coli* C25. Thus, it appears that the host immune system must first be "primed" by the indigenous GI microflora before a later second "stimulation" by *P. acnes* vaccine activates macrophages sufficient to inhibit bacterial translocation.

Interestingly, *P. acnes* vaccination does not reduce *E. coli* translocation to the MLN even if adult germfree mice are monoassociated with *E. coli* C25 or colonised with the whole indigenous GI microflora for 8 weeks prior to *P. acnes* vaccination (*Berg* and *Itoh*, 1986). However, if the germfree mice are colonised with the whole indigenous GI microflora within 1 week after birth, then a subsequent *P. acnes* vaccination at 8 weeks of age inhibits *E. coli* C25 translocation in these mice. Thus, exposure to the indigenous microflora is not a prerequisite for splenomegaly induced by *P. acnes* vaccination but is required for inhibition of bacterial translocation.

Other investigators also have reported that the indigenous microflora is

required for the normal development of cell-mediated immunity. *Starling* and *Balish* (1981) found that germfree rats have fewer pulmonary macrophages than conventional rats and that exposure to the indigenous microflora increases alveolar macrophage proliferation and activity. There also are reports that colonisation of germfree animals with the indigenous microflora increases the activities of peritoneal macrophages (*Morland* and *Midtvedt*, 1984) and natural killer cells (*Bartizal* et al., 1983). *Pabst* et al. (1982) suggest that human monocytes do not function optimally unless they have been exposed to the indigenous microflora.

Scott (1972), *Bash* (1978), *Wells* and *Balish* (1979), and *Johnson* and *Balish* (1980) found that prior exposure to the indigenous microflora influences the effectiveness of *P. acnes* vaccination. *P. acnes* given i.p. to conventional rats stimulated the splenic PFC response to sheep erythrocytes whereas *P. acnes* vaccination inhibited this spleen PFC response in germfree rats or gnotobiotic rats monoassociated with *P. acnes*. It does not seem unusual that the indigenous microflora affects the development of the host immune response since the indigenous microflora also is known to profoundly influence the anatomic and physiologic development of the host.

CONCLUSION

Animal models have been developed whereby bacterial components can either promote or inhibit bacterial translocation from the GI tract. One i.p. injection of endotoxin causes histologic damage to the intestinal mucosa and promotes the translocation of indigenous bacteria from the GI tract to the MLN. Inhibition or inactivation of xanthine oxidase or dehydrogenase activities reduces the extent of endotoxin-induced mucosal damage and also decreases bacterial translocation. Thus, a primary mechanism of endotoxin-induced bacterial translocation appears to be the production of xanthine oxidase-generated oxidants which damage the gut mucosa. It also is possible that the translocating bacteria and their products (e.g. endotoxin) further exacerbate the initial mucosal injury caused by endotoxic shock. Studies are in progress to delineate the relationships among endotoxic shock, mucosal injury, mucosal permeability, the translocation of bacteria across the mucosal barrier, and lethal sepsis.

In order to design therapeutic regimens to prevent or decrease bacterial translocation, much more knowledge is required concerning the role of the host immune defence against bacterial translocation. The results described here demonstrating that *P. acnes* vaccination inhibits the translocation of *E. coli* from the GI tract to the MLN suggest that macrophages are important immune effector cells in the host defence. The fact that macrophages can be activated non-specifically to inhibit the translocation of a variety of bacteria is of practical significance, since it cannot be predicted with certainty which of the many indigenous bacterial species will translocate from the GI tract in any given clinical situation. The described studies utilising athymic mice suggest that T-cell mediated immunity also is important in the immune defence against translocation. Studies are in progress to determine the relative roles in preventing bacterial translocation of various compartments of the host immune system, such as secretory

immunity on mucosal surfaces, cell-mediated immunity (macrophages, neutrophils, and T-cell subpopulations), and systemic immunity (serum IgG and IgM).

Bacterial translocation involves complex interactions between the host defence mechanisms and the abilities of bacteria to translocate mucosal barriers and to survive in hostile environments. Delineation of the immune mechanisms important in inhibiting bacterial translocation will provide information for devising rational approaches for the control of opportunistic infections originating from the GI tract in debilitated patients, such as those with endotoxaemia, thermal injury, trauma, and immunosuppressive disorders such as AIDS.

LITERATURE

Baker, J.W., Deitch, E.A., Berg, R.D., and Ma, L.: Hemorrhagic shock impairs the mucosal barrier resulting in bacterial translocation from the gut and sepsis. Surg. Forum 37, 73-74 (1987).

Baker, J.W., Deitch, E.A., Berg, R.D., and Specian, R.D.: Hemorrhagic shock induces bacterial translocation. J. Trauma 28, 896-906 (1988).

Bartizal, K.F., Salkowski, C., and Balish, E.: The influence of a gastrointestinal microflora on natural killer cell activity. RES J. Reticulendothelial Soc. 33, 381-390 (1983).

Bash, J.A.: Suppression of rat T-cell proliferation by *Corynebacterium parvum*: T-cell requirement for induction. RES. J. Reticuloendothelial Soc. 23, 63-73 (1978).

Berg, R.D.: Inhibition of *Escherichia coli* translocation from the gastrointestinal tract by normal cecal flora in gnotobiotic or antibiotic-decontaminated mice. Infect. Immun. 29, 1073-1081 (1980a).

Berg, R.D.: Mechanisms confining indigenous bacteria to the gastrointestinal tract. Amer. J. Clin. Nutr. 33, 2472-2484 (1980b).

Berg, R.D.: The translocation of normal flora bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs. Microecol. Ther. 11, 27-34 (1981a).

Berg, R.D.: Factors influencing the translocation of bacteria from the gastrointestinal tract. In: Recent Advances in Germfree Research (Eds.: Sasaki, S., Ogawa, A., and Hashimoto, K.). Tokai University Press, Tokyo, 411-418 (1981b).

Berg, R.D.: Promotion of the translocation of enteric bacteria from the gastrointestinal tracts of mice by oral treatment with penicillin, clindamycin, or metronidazole. Infect. Immun. 33, 854-861 (1981c).

Berg, R.D.: Bacterial translocation from the gastrointestinal tracts of mice receiving immunosuppressive chemootherapeutic agents. Curr. Microbiol. 8, 285-292 (1983a).

Berg, R.D.: Translocation of the indigenous bacteria from the intestinal tract. In: The Intestinal Microflora in Health and Disease (Ed.: Hentges, D.). Academic Press, NY, 333-352 (1983b).

Berg, R.D.: Bacterial translocation from the intestines. Expt. Animals 34, 1-16 (1985).

Berg, R.D. and Garlington, A.W.: Translocation of certain indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs in a gnotobiotic mouse model. Infect. Immun. 23, 403-411 (1979).

Berg, R.D. and Itoh, K.: Bacterial translocation from the gastrointestinal tract-Immunologic aspects. Microecol. Ther. 16, 131-145 (1986).

Berg, R.D. and Owens, W.E.: Inhibition of translocation of viable *Escherichia coli* from the gastrointestinal tract of mice by bacterial antagonism. Infect. Immun. 25, 820-827 (1979a).

Berg, R.D. and Owens, W.E.: Inhibition of translocation of viable *Escherichia coli* from the gastrointestinal tract to the mesen-

teric lymph nodes and other organs in a gnotobiotic mouse model. Infect. Immun. 23, 403-411 (1979b).

Braquet, P.L., Touqui, L., Shen, T.Y., and Vargaftig, B.B.: Perspectives in platelet-activating factor research. Pharmacol. Rev. 39, 97-145 (1987).

Cahill, C.J., Pain, J.A., and Bailey, M.E.: Bile salts, endotoxin and renal function in obstructive jaundice. Surg. Gynecol. Obstet. 165, 519-521 (1987).

Carrico, C.J., Meakins, J.L., Marshall, J.C., Fry, D., and Maier, R.V.: Multiple organ failure syndrome. Arch. Surg. 121,196-208 (1986).

Collins, F.M. and Scott, M.T.: Effects of *Corynebacterium parvum* treatment on the growth of *Salmonella enteritidis* in mice. Infect. Immun. 9, 863-869 (1974).

Cuevas, P. and Fine, J.: Production of fatal endotoxin shock by vasoactive substances. Gastroenterology 64, 285-291 (1973).

Deitch, E.A. and Berg, R.D.: Endotoxin but not malnutrition promotes bacterial translocation of the gut flora in burned mice. J. Trauma 27, 161-166 (1987).

Deitch, E.A., Berg, R., and Specian, R.: Endotoxin promotes the translocation of bacteria from the gut. Arch. Surg. 122, 185-190 (1987a).

Deitch, E.A., Bridges, W.R., Ma, J.W., Ma, L., Berg, R.D., and Specian, R.D.: The obstructed intestine as a resevoir for systemic infection. Arch. Surg. 159, 394-401 (1990a).

Deitch, E.A., Kemper, A.C., Specian, R.D., and Berg, R.D.: A study of the relationships between bacterial translocation, gut-origin sepsis and survival in a model of systemic inflammation. J. Trauma 32, 141-147 (1992).

Deitch, E.A., Ma, J-W., Ma, L., Berg, R., and Specian, R.: Endotoxin-induced bacterial translocation: A study of mechanisms. Surgery 106, 292-300 (1989a).

Deitch, E.A., Ma, L., Ma, J-W., Grisham, M., Granger, N., Specian, R., and Berg, R.: Inhibition of endotoxin-induced bacterial translocation in mice. J. Clin. Invest. 84, 36-42 (1989b).

Deitch, E.A., Maejima, K., and Berg, R.D.: Effect of oral antibiotics and bacterial overgrowth on the translocation of the GI tract microflora in burned rats. J. Trauma 25, 385-392 (1985).

Deitch, E.A., Sittig, K., MA, L., Berg, R., and Specian, R.: Obstructive jaundice promotes bacterial translocation from the gut. Amer. J. Surg. 159, 79-84 (1990b).

Deitch, E.A., Winterton, J., MA, L., and Berg, R.: The gut as a portal of entry for bacteremia: The role of protein malnutrition. Ann. Surg. 205, 681-692 (1987b).

Fuller, R. and Jayne-Williams, D.J.: Resistance of the fowl (*Gallus domesticus*) to invasion by its intestinal flora. II. Clearance of translocated intestinal bacteria. Res. Vet. Sci. 11, 368-374 (1970).

Granger, D.N., Hollwarth, M.E., and Parks, D.A.: Ischemia-reperfusion injury: Role of oxygen-derived free radicals. Acta Physiol. Scand. 548 (Suppl.), 47-64 (1986).

Granger, D.N., Rutili, G., and McCord, J.M.: Role of superoxide radicals in intestinal ischemia. Gastroenterology 8, 22-29 (1981).

Hassid, W.Z., Joslyn, M.A., and McCready, R.M.: The molecular constitution of an insoluble polysaccharide from yeast, *Saccharomyces cerevisiae*. J. Amer. Chem. Soc. 63, 295-298 (1941).

Herbert, J.C., Gamelli, R.L., Foster, R.S. Jr., Chalmer, B.J., and Davis, J.H.: Improved survival after pneumococcus in splenectomized and nonsplenectomized mice with *Corynebacterium parvum*. Arch. Surg. 118, 328-332 (1983).

Jacobs, D.M. and Morrison, D.C.: Inhibition of the mitogenic response of LPS in mouse spleen cells by polymyxin-B. J. Immunol. 118, 21-27 (1977).

Johnson, W.J. and Balish, E.: Macrophage function in germ-free, athymic (nu/nu), and conventional flora (nu/+) mice. J. Reticuloendothelial Soc. 28, 55-64 (1980).

Keller, R. and Engley, F.B., Jr.: Fate of bacteriophage particles introduced into mice by various routes. Proc. Soc. Exp. Biol. Med. 98, 577-580 (1958).

Li, M., Specian, R.D., Berg, R.D., and Deitch, E.A.: Effects of protein malnutrition and endotoxin on the intestinal mucosal barrier to the translocation of indigenous flora in mice. Parenteral and Enteral Nutr. 13, 572-578 (1989).

Maejima, K., Deitch, E.A., and Berg, R.D.: Promotion by burn stress of the translocation of bacteria from the gastrointestinal tracts of mice. Arch. Surg. 119, 166-172 (1984a).

Maejima, K., Deitch, E.A., and Berg, R.D.: Bacterial translocation from the gastrointestinal tract of rats receiving thermal injury. Infect. Immun. 43, 6-10 (1984b).

Miyata, H., Nomoto, K., and Takeya, K.: Characteristics of resistance to *Listeria monocytogenes* enhanced by *Corynebacterium parvum* in mice. Immunology 40, 33-39 (1980).

Morehouse, J., Specian, R., Stewart, J., and Berg, R.D.: Promotion of the translocation of indigenous bacteria of mice from the GI tract by oral ricinoleic acid. Gastroenterology 91, 673-682 (1986).

Morland, B. and Midtvedt, T.: Phagocytosis, peritoneal influx, and enzyme activities in peritoneal macrophages from germfree, conventional, and ex-germfree mice. Infect. Immun. 44, 750-752 (1984).

Morrison, D.C. and Ryan, J.L.: Endotoxins and disease mechanisms. Ann. Rev. Med. 38, 417-432 (1987).

Nolan, J.P.: Endotoxin, reticuloendothelial function, and liver injury. Hepatolgy 1, 458-465 (1981).

Owens, W.E. and Berg, R.D.: Bacterial translocation from the gastrointestinal tract of athymic (nu/nu) mice. Infect. Immun. 24, 308-312 (1980).

Owens, W.E. and Berg, R.D.: Bacterial translocation from the gastrointestinal tracts of thymectomized mice. Curr. Microbiol. 7, 169-174 (1982).

Pabst, M.J., Hedegaard, H.B., and Johnston, R.B.: Altered human monocytes require exposure to bacterial products to maintain an optimal oxygen radical response. J. Immunol. 128, 123-128 (1982).

Parks, D.A. and Granger, D.N.: Xanthine oxidase: Biochemistry, distribution and physiology. Acta Physiol. Scand. 548 (Suppl.), 87-100 (1986).

Parks, D.A., Bulkley, G.B., Granger, D.N., Hamilton, S.R., and McCord, J.M.: Ischemic injury in the cat small intestine: Role of superoxide radicals. Gastroenterology 82, 9-15 (1982a).

Parks, D.A., Grogaard, B., and Granger, D.N.: Comparison of partial and complete arterial occlusion models for studying intestinal ischemia. Surgery 92, 896-901 (1982b).

Penn, R.L., Maca, R.D., and Berg, R.D.: Leukemia promotes the translocation of indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs. Microecol. Ther. 15, 85-91 (1986).

Ravin, H.A. and Fine, J.: Biological implications of intestinal endotoxins. Fed. Proc. 21, 65-68 (1962).

Scott, M.T.: Depression of delayed-type hypersensitivity by *Corynebacterium parvum*. Mandatory role of the spleen. Cell. Immunol. 13, 251-263 (1972).

Smith, S.M. , Grisham, M.B., Manci, E.A., Granger, D.N., and Kvietys, P.R.: Gastric mucosal injury in the rat: Role of iron and xanthine oxidase. Gastroenterology 92, 950-956 (1987).

Starling, J.R. and Balish, E.: Lysosomal enzyme activity in pulmonary alveolar macrophages from conventional, germfree, monoassociated, and conventionalized rats. J. Reticuloendothelial Soc. 30, 497-505 (1981).

Steffen, E.K. and Berg, R.D.: Relationship between cecal population levels of indigenous bacteria and translocation to the mesenteric lymph nodes. Infect. Immun. 39, 1252-1259 (1983).

Steffen, E.K., Berg, R.D., and Deitch, E.A.: Comparison of the translocation rates of various indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes. J. Infect. Dis. 157, 1032-1037 (1988),

Stinnett, J.D., Morris, M.J., and Alexander, J.W.: Macrophage activation and increased

resistance to infection in immunosuppressed mice treated with *Corynebacterium parvum* vaccine or pyran copolymer. J. Reticuloendothelial Soc. 25, 525-531 (1979).

Sun, X.M. and Hsueh, W.: Bowel necrosis induced by tumor necrosis factor in rats mediated by platelet-activating factor. J. Clin. Invest. 81, 328-1331 (1988).

Ward, W.R. and Rajagopolan, K.V.: Purification and properties of the NAD^+-dependent (type D) and O_2-dependent (type O) form of rat liver xanthine dehydrogenase. Arch. Biochem. Biophys. 172, 354-364 (1976).

Wells, C.L. and Balish, E.: Immune response modulation by colonization of germfree rats with *Propionibacterium acnes*. Infect. Immun. 26, 473-478 (1979).

Wilson, R.F.: Special problems in the diagnosis and treatment of surgical sepsis. Surg. Clinics N. Amer. 65, 965-989 (1985).

Wolochow, H., Hildebrand, G.J., and Lamanna, C.: Translocation of microorganisms across the intestinal wall of the rat: effect of microbial size and concentration. J. Infect. Dis. 116, 523-528 (1966).

Wooles, W.R. and DiLuzio, N.R.: Reticuloendothelial function and the immune response. Science 142, 1078-1080 (1962).

Old Herborn University Seminar Monograph 4:
Host-microflora interactions in the first years after birth.
Editors: Peter J. Heidt, Rial D. Rolfe, Volker Rusch, and Dirk van der Waaij.
Institute for Microbiology and Biochemistry, Herborn-Dill, Germany: 87-92 (2002).

THE IgA SUBCLASS DISTRIBUTION OF ANTIBODIES AGAINST LIPOPOLYSACCHARIDES FROM INTESTINAL BACTERIA

LENNART HAMMARSTRÖM[1], ANNA JANSSON[1], PER-ERIK ENGSTRÖM[1], BENGT LAVÖ[2], NILS FELTELIUS[2], LARS KNUTSON[3], LARS KANERUD[4], ROGER HÄLLGREN[2], and C.I. EDVARD SMITH[1]

[1]Department of Clinical Immunology and Centre for Biotechnology, Karolinska Institute at Huddinge Hospital, Huddinge, Sweden,
[2]Department of Internal Medicine, University Hospital, Uppsala, Sweden,
[3]Department of Surgery, University Hospital, Uppsala, Sweden, and
[4]Department of Internal Medicine, Karolinska Institute at Södersjukhuset, Stockholm, Sweden.

SUMMARY

Using a novel technique for sampling of jejunal perfusion fluid, the IgA levels and subclass distribution of secreted antibodies against bacterial lipopolysaccharides were analysed in healthy controls and patients with various diseases. The highest level of jejunal fluid IgA was highest in patients with coeliac disease whereas the highest serum IgA levels were found among patients with ankylosing spondylitis.

Jejunal antibodies against lipopolysaccharide from *E. coli* were to a large extent of the IgA2 subclass, regardless of patient group whereas serum IgA antibodies from the corresponding patients were almost exclusively of the IgA1 subclass. IgA2 antibodies against lipopolysaccharide from *B. fragilis* were also found in jejunal secretions, again in contrast to the pattern found in serum.

These findings support the notion of a dichotomy between the secretory IgA system and the serum IgA system and suggests that these two systems are independently regulated.

INTRODUCTION

The mucosal immune system develops independently of the systemic immune system and maturity of the secretory IgA system is reached already at an early age whereas adult levels of serum IgA may not be seen until adolescence. During development there is an active interplay between the bacterial content of the gut and the differentiating immune system where the former may influence the subsequent immunoglobulin class pattern.

Human IgA may be subdivided into two biochemically distinct subclasses, each encoded by a separate gene (*Feinstein* and *Franklin*, 1966; *Vaerman* and *Heremans*, 1966). It has as yet not been fully elucidated whether these subclasses are also different with regard to biological and functional properties.

Previous studies have suggested that cells secreting IgA1 are far more abundant in the jejunum than cells producing IgA2 (*Brandtzaeg* et al., 1986), a finding which also reflected in the subclass distribution of the secreted antibodies (*Delacroix* et al., 1982).

The subclass distribution of specific

Table 1: IgA concentrations in serum and jejunal fluid[a]

Patient category	n	Serum IgA (g/l)	n	Jejunal IgA (mg/l)
Healthy controls	7	2.2 (0.9-4.6)	9	14.8 (5.2-56.5)
Coeliac disease[b]	24	2.1 (0.5-3.9)	30	24.4 (4.8-136.3)
Ankylosing spondylitis	22	3.5 (0.9-10.3)	21	18.9 (3.3-60.0)
Rheumatoid arthritis	17	2.2 (0.5-4.7)	19	11.9 (3.2-42.5)

a. Results are given as mean levels of IgA (range).
b. Patients with IgA deficiency excluded from the calculation.

serum IgA antibodies against a number of antigens have been described to date. In most instances, a marked dominance of IgA1 is seen. Far less is however known about the subclass distribution of secreted antibodies although limited studies in saliva and breast milk (for review see *Mestecky* and *Russel*, 1986) suggests that some IgA2 antibodies may be found against selected bacterial antigens.

The purpose of this study was to analyse the IgA subclass distribution of antibodies against bacterial lipopolysaccharides in serum and jejunal perfusion fluid in patients with various disorders.

MATERIALS AND METHODS

Patient samples

Jejunal perfusion was performed as described previously in detail (*Knutson* et al., 1989). The samples were collected and stored at -70°C until used. Sera were obtained simultaneously and similarly stored until assayed. Samples were obtained from 9 normal control subjects, 30 patients with coeliac disease, 19 patients with rheumatoid arthritis and 22 patients with ankylosing spondylitis. Total IgA in jejunal fluid was measured using alpha-specific rabbit antibodies in a sandwich ELISA (Dakopatts, Copenhagen, Denmark). Two mM of a protease inhibitor (phenylmethylsulfonylfluoride, Sigma chemical Co., MO, USA) was added to the jejunal fluid in all experiments in order to avoid degradation of IgA. Serum levels of IgA were measured by nephelometry.

ELISA for IgA subclass distribution of specific antibodies

Specific serum and jejunal fluid antibodies of the IgA class were measured at a concentration of 10 µg/ml of IgA. The levels were measured in single wells in ELISA as has been described in detail previously (*Engström* et al., 1990). The antigens (lipopolysaccharide from *E. coli* and *B. fragilis* which were both gifts from Dr. A. Weintraub, Dept. of Microbiology, Huddinge Hospital, Huddinge, Sweden) were coated with bicarbonate buffer at a concentration of 2 µg/ml. An alkaline phosphatase conjugated rabbit anti human IgA antiserum (Dakopatts, Copenhagen, Denmark) was used for assessment of total IgA levels whereas monoclonal reagents were used for subclass determinations (anti-IgA1 and anti-IgA2 both from Nordic Laboratories, Tilburg, The Netherlands) followed by rabbit anti-mouse antibodies (Dakopatts) and alkaline phophatase conjugated sheep $F(ab)_2$ anti-rabbit antiserum.

A monoclonal chimeric IgA2 antibody directed against the hapten NP

Table 2: IgA antibodies against bacterial lipopolysaccharides[a]

	n	Antigen	Serum	Jejunal fluid
Controls	5	*E. coli* LPS	0.24 (0.13-0.36)	1.80 (0.78-2.62)
Patients	28	*E. coli* LPS	0.25 (0.06-0.53)	1.31 (0.25-2.86)
Controls	5	*B. fragilis* LPS	0.12 (0.02-0.19)	1.89 (0.32-2.65)
Patients	28	*B. fragilis* LPS	0.18 (0.03-0.41)	0.97 (0.05-2.77)

a. Results are given as mean net absorbance (range) after 40 minutes.

(*Brüggeman* et al., 1987) served as standard for semiquantitative measurements of specific antibodies of the IgA class and IgA2 subclass (tested on separate plates coated with NP-BSA, a gift from Prof. O. Mäkelä, Dept. of Bacteriology and Immunology, University of Helsinki, Helsinki, Finland) which were run in parallel as has been described previously (*Engström* et al., 1990). Serum and saliva from donors with IgA class or subclass deficiency served as specificity controls as described previously (*Engström* et al., 1988, 1990).

RESULTS

Concentrations of IgA in serum and jejunal fluid

The levels of both serum IgA and jejunal fluid IgA differed markedly between patient groups, serum levels being highest in the patients with ankylosing spondylitis (Table 1). Four coeliac patients were deficient in serum IgA; these patients also lacked IgA in jejunal fluid (<0.01 µg/ml).

Subclass distribution of anti-lipopolysaccharide antibodies

Selection of samples to be tested for IgA subclass distribution was based on the presence of concentrations of IgA in the jejunal fluid of more than 10 µg/ml. Samples from 5 normal control subjects, 10 patients with coeliac disease, 5 patients with rheumatoid arthritis and 12 patients with pelvospondylitis were assayed in two separate experiments where the samples were diluted to give a final concentration of 10 µg/ml. The corresponding serum samples were similarly diluted.

In a first set of experiments, the subclass pattern of serum IgA antibodies against lipopolysaccharide from *E. coli* and *B. fragilis* was determined. At the level tested, only IgA1 antibodies were detected (data not shown). The pattern in jejunal fluid samples was markedly different. First, levels of specific anti-lipopolysaccharide antibodies, as determined by absorbance values, were around tenfold higher in jejunal fluid as compared to serum (Table 2). Second, the subclass pattern was quite distinct, with major contributions from IgA2, again as determined by absorbance levels, in all patient categories without any major deviance in any particular disease (Table 3).

Proportion of anti-lipopolysaccharide antibodies

The ELISA is not a quantitative method and the above levels of absorbance may be misleading in terms of true antibody levels. We have previously developed a semiquantitative

Table 3: IgA subclass distribution of anti-lipopolysaccharide antibodies in jejunal perfusion fluid[a]

Patient category	n	*E. coli* LPS IgA1	*E. coli* LPS IgA2	*B. fragilis* LPS IgA1	*B. fragilis* LPS IgA2
Healthy controls	5	1.91	0.95	1.07	0.89
Coeliac disease	12	1.35	0.74	1.02	0.64
Ankylosing spondylitis	11	1.48	0.76	1.14	0.52
Rheumatoid arthritis	5	1.50	0.78	1.02	0.45

a. Results are given as mean net absorbance after 30 minutes.

method for IgA and IgA2 antibodies in serum and secretions based on comparison from a standard curve established with the aid of a human monoclonal antibody directed against NP. Thus, in one experiment, an attempt was made to quantify the antibodies found in jejunal fluid. As evident in Table 4, a fair proportion of antibodies against bacterial lipopolysaccharides were of the IgA2 subclass. Antibodies against the two antigens tested constituted a few percent of the total amount of IgA antibodies present in jejunal fluid (Table 4). Since the mean total level of IgA was in jejunal fluid was around 15 µg/ml, approximately 30 ng/ml of specific anti-*E. coli* lipopolysaccharide antibodies are normally found in jejunal fluid.

DISCUSSION

Concentrations of specific IgA antibodies in jejunal secretions are difficult to determine due to technical reasons. The abundance of proteolytic enzymes may degrade the antibodies during processing and the presence of enzyme inhibitors both during the jejunal lavage and the subsequent assays is a necessary precaution. Second, hitherto available systems for sampling (open or semi-open techniques) frequently suffer from proximal or distal contamination thus introducing errors in the evaluation of the samples. Furthermore, such techniques often require high perfusion rates and the low and variable recovery of perfusion fluid results in a low precision in the analysis of IgA. The recent development of an adequate technique for sampling has allowed the reliable measurement of complement factors (*Ahrenstedt* et al., 1990) and antibodies (unpublished results) in jejunal lavage fluid.

Specific antibodies in jejunal fluid against bacterial lipopolysaccharides constituted a few percent of total IgA, markedly higher than in serum. This suggests that the local bacterial load influences the level of specific antibodies. The observed levels of antibodies also indicate that gastrointestinal bacteria induce local immunity rather than tolerance.

The subclass distribution of the specific anti-lipopolysaccharide antibodies shows a dominance of IgA2. This is not a reflection of the total amount of IgA1 and IgA2 synthesised since there is normally a predominance of IgA1 producing cells in the jejunum (*Brandtzaeg* et al., 1986). Our findings thus confirm and extend previous findings (*Moldoveanu* et al., 1987) on the subclass distribution of anti- lipopolysaccharide antibodies in secretions. Since the former study was performed using saliva as a source of antibodies,

Table 4: Quantitative estimate of specific IgA and IgA2 antibodies in jejunal perfusion fluid[a]

E. coli LPS		*B. fragilis* LPS	
IgA (ng/ml)	IgA2 (%[b])	IgA (ng/ml)	IgA2 (%)
20.9	62	9.6	82

a. Results are given as mean levels in the 15 randomly chosen subjects tested (3 healthy controls, 7 patients with coeliac disease, 2 patients with ankylosing spondylitis and 3 patients with rheumatoid arthritis; all tested at a fixed concentration of 10 µg/ml of IgA).
b. Results are given as mean % of specific IgA2 antibodies.

the finding of a similar subclass pattern in jejunal fluid supports the notion of a common secretory immune system with homing of IgA producing cells in distal mucosal tissues after initial priming in the gut (for review see *Mestecky* and *McGhee*, 1987).

Since protein antigens mainly induce IgA1 in secretions (for review see *Mestecky* and *Russel*, 1986),the IgA2 response to polysaccharide antigens appears to reflect a distinct regulatory pathway. This mechanism, although still elusive with regard to its molecular basis, is reminiscent of the subclass restriction of IgG antibodies where polysaccharide antigens mainly induce IgG2 whereas protein antigens preferentially elicit IgG1 antibodies (for review see *Hammarström* and *Smith*, 1986).

It remains to be determined whether the mucosal response to different antigens (or epitopes on these antigens) is identical to that of the systemic immune response. Our results suggest that responses may be different at least in terms of subclass composition. The continued utilisation of the sampling technique employed in this paper may allow a resolution of this question.

ACKNOWLEDGEMENTS

This work was supported by the Swedish Medical Research Council and the Swedish Life Insurance Companies Trust for Medical Research.

LITERATURE

Ahrenstedt, Ö., Knutson, L., Nilsson, B., Nilsson-Ekdahl, K., Odlind, B., and Hällgren, R.: Enhanced local production of complement components in the small intestines of patients with Crohn´s disease. N. Engl. J. Med. 322, 1345-1349 (1990).

Brandtzaeg, P., Kett, K., Rognum, T.O., Söderström, R., Björkander, J., Söderström, T., Petrusson, B., and Hanson, L.Å.: Distribution of mucosal IgA and IgG subclass-producing immunocytes and alterations in various disorders. Monogr. Allergy 20, 179-194 (1986).

Brüggemann, M., Williams, G.T., Bindon, C.I., Clark, M.R., Walker, M.R., Jefferis, R., Waldmann, H., and Neuberger, M.S.: Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. J. Exp. Med. 166, 1351-1360 (1987).

Delacroix, D.L., Dive, C., Rambaud, J.C., and Vaerman, J.P.: IgA subclasses in various secretions and in serum. Immunol. 47, 383-385 (1982).

Engström, P-E., Norhagen, G., Smith, C.I.E., Söder, P-Ö., and Hammarström, L.: An enzyme-linked immunosorbent assay for the determination of the IgA subclass distribution of antigen-specific antibodies. J. Immunol. Meth. 115, 45-53 (1988).

Engström, P-E., Norhagen, G., Bottaro, A., Carbonara, A.O., Lefranc, G., Steinitz, M., Söder, P-Ö., Smith, C.I.E., and Hammarström, L.: Subclass distribution of antigen-specific antibodies in normal donors and individuals with homozygous Cα1 or Cα2 gene deletions. J. Immunol. 145, 109-116, 1990.

Feinstein, D. and Franklin, E.C.: Two antigenically distinguishable subclasses of human γA myeloma proteins differing in their heavy chains. Nature 212, 1496-1498 (1966).

Hammarström, L. and Smith, C.I.E.: IgG subclasses in bacterial infections. Monogr. Allergy 19, 122-133 (1986).

Knutson, L., Odlind, B., and Hällgren, R.: A new technique for segmental jejunal perfusion in man. Am. J. Gastroenterol. 84, 1278-1284 (1989).

Mestecky, J. and McGhee, J.R.: Immunoglobulin A (IgA): Molecular and cellular interactions involved in IgA biosynthesis and immune response. Adv. Immunol. 40, 153-245 (1987).

Mestecky, J. and Russell, M.W.: IgA subclasses. Monogr. Allergy 19, 277-301 (1986).

Moldoveanu, Z., Brown, T.A., Ventura, M.T., Michalek, S.M., McGhee, J.R., and Mestecky, J.: IgA subclass responses to lipopolysaccharide in humans. In: Recent advances in mucosal immunology (Eds.: Mestecky, J., McGhee, J.R., Bienenstock, J., and Orga, P.L.). Plenum Publishing Corporation, New York, 1199-1205 (1987).

Vaerman, J.P., and Heremans, J.F.: Subgroups of human immunoglobulins A based on differences in the alpha polypeptide chains. Science 153, 647-649 (1966).

Old Herborn University Seminar Monograph 4:
Host-microflora interactions in the first years after birth.
Editors: Peter J. Heidt, Rial D. Rolfe, Volker Rusch, and Dirk van der Waaij.
Institute for Microbiology and Biochemistry, Herborn-Dill, Germany: 93-98 (2002).

THE INTERPLAY BETWEEN THE IMMUNE DEFENCE IN THE NEONATE AND THE FLORA COLONISING THE GUT AFTER BIRTH

LARS Å. HANSON[1], MIRJANA HAHN-ZORIC[1], RIFAT ASHRAF[5], BARBRO CARLSSON[1], SHAKILA ZAMAN[5], JOHAN KARLBERG[2], SHAUKAT R. KHAN[4], BO S. LINDBLAD[3], and FEHMIDA JALIL[5]

[1]Department of Clinical Immunology, and [2]Department of Anatomy, University of Göteborg, Göteborg, Sweden, [3]Department of Paediatrics at Karolinska Institute, St. Göran's Children's Hospital, Stockholm, Sweden, and [4]Departments of Paediatrics and [5]Department of Social and Preventive Paediatrics, King Edward Medical College, Lahore, Pakistan.

SUMMARY

Specific and non-specific host defence in the new-born is of great importance for preventing many of the microbes, colonising the skin and various mucous membranes from causing infections. The passively transferred specific IgG antibodies from the mother as well as defence factors in the breast milk are important. On the other hand, the microbial flora exposing the mother and the infant will influence and direct the immune response of the foetus and new-born. This presentation will discuss the antibody response of the young to microbes with special reference to the possible effect of anti-idiotypic antibodies from the mother via the placenta or the milk. The differences in intestinal colonisation seen between Swedish and Pakistani infants is presented in relation to the differences in the mode of feeding.

A FOETAL AND NEONATAL ANTIBODY RESPONSE IN MAN POSSIBLY INDUCED BY ANTI-IDIOTYPIC ANTIBODIES FROM THE MOTHER

In some instances it has been noted that new-borns have antibodies that have not come from the mother. We have noticed secretory IgA and IgM (SIgA and SIgM) antibodies to appear in new-borns' saliva both against *E. coli* and poliovirus antigens (*Mellander* et al., 1984, 1986). These antibodies also appeared in new-borns of mothers with hypogammaglobulinaemia and IgA deficiency demonstrating that they must have been produced by the foetus (*Mellander* et al., 1986; *Hahn-Zoric* et al., 1992). The antibodies against the poliovirus in Swedish new-borns was of special interest since efficient vaccination using only inactivated poliovirus has eliminated poliomyelitis and there are, with very few exceptional imports, no wild or vaccine poliovirus in the country. Thus it is very unlikely that these foetuses can have been exposed to poliovirus antigens.

We have assumed that the antigenic stimulus of the foetus could be anti-idiotypic antibodies from the mother. In several assays we have found evidence for the presence of such anti-anti-poliovirus antibodies in the immunoglobulin preparation given to the

mothers with hypogammaglobulinaemia as a prophylaxis against infections (*Hanson* et al., 1989a; *Hahn-Zoric* et al., 1993). If our assumption is correct it means that the IgG antibodies passively received by the foetus from the mother may not only passively protect, but may even actively prime the immune system of the offspring. Such effects of anti-idiotypic antibodies given directly to new-born mice, or via the mother animal reaching the offspring through the milk have been demonstrated against bacterial as well as viral antigens (*Stein* and *Söderström*, 1984; *Okamoto* et al., 1989)

In a preliminary study we found some evidence that breast-fed infants responded better to parenteral diphtheria and tetanus toxoid and oral poliovirus vaccine than formula-fed infants (*Hahn-Zoric* et al., 1990). The SIgA responses in saliva and IgM in stool were higher at 3-4 months of age, whereas the serum IgG antibody responses to diphtheria toxoid and poliovirus were higher at 20-40 months of age. We have proposed that these enhanced responses might be the result of anti-idiotypic antibodies in milk priming the lymphoid system of the breast-fed infants. Some data indicate that human milk can contain anti-idiotypic antibodies (*Hanson* et al., 1989a; *Hahn-Zoric* et al., 1993).

Presumably the exposure of the mother to microbes will direct her immune response and her level of the corresponding idiotypes - anti-idiotypes, which will be transferred to the foetus. Little is presently known about whether stimulation or inhibition will ensue. We did not observe any obvious difference in the content in saliva from Swedish and Pakistani new-borns of SIgA and IgM antibodies to *Escherichia coli* O antigens (*Mellander* et al., 1985). Still the Pakistani new-borns and their mothers studied must be much more heavily exposed to such intestinal bacteria and those infants increase their salivary content of SIgA antibodies to *E. coli* O antigens quickly after birth (*Mellander* et al., 1985).

INTESTINAL COLONISATION OF PAKISTANI AND SWEDISH NEW-BORNS WITH GRAM-NEGATIVE AEROBES

Pakistani infants were significantly earlier colonised in the gut with Gram-negative aerobes than Swedish infants (*Adlerberth* et al., 1990). This was true whether they were delivered at home or at a hospital, whether through vaginal delivery or through caesarean section. The Pakistani infants had a more variable flora than the Swedish infants, who mainly had *E. coli*, whereas *Enterobacter*, *Citrobacter* and *Klebsiella* were more common in the Pakistani infants, although less so if they were breast-fed than if given others foods (*Adlerberth* et al., 1990).

The binding to a colon epithelial cell line of some colonising *E. coli* could occasionally be inhibited by a meconium extract. This was seen for strains with adhesins which agglutinated red blood cells of blood group p (-) without being inhibited by mannose (*Hanson* et al., 1989b). It is possible that this mechanism can play a role for the capacity of the new-born to handle some of the bacteria colonising the gut directly after birth. It is likely that the effect is due to the presence of receptor analogues in the meconium, just as we have shown receptor analogues in human milk which can prevent adhesion of pneumococci and *Haemophilus influenzae* to retropharyngeal cells (*Andersson* et al., 1986).

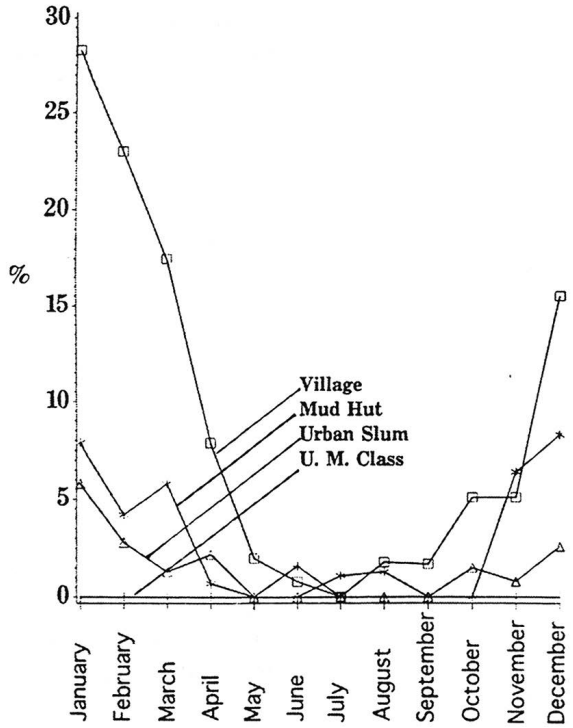


Figure 1: Exclusive breastfeeding in a prospective follow up of four different population groups (ages from 1 to 6 months) in and around Lahore, Pakistan, in relation to the month of the year.

We have proposed that human milk supports the health of the breast-fed infant not only by the anti-adhesive effect of antibodies and receptor analogues, but also via a number of components in milk which are anti-inflammatogenic (*Goldman* et al., 1986). Thus lactoferrin inhibits complement, the secretory IgA antibodies do not activate complement and the lysozyme limits chemotaxis and inflammatogenic activity of neutrophils. Milk lipids inhibit neutrophil superoxide production. The superoxide is also counteracted by the antioxidants in milk, alfa-tocopherol, cysteine and ascorbic acid and is destroyed by the milk catalase and glutathione peroxidase, etc. These capacities may be useful for instance for dampening the effect of endotoxin from the gut flora on the neonate's intestine.

In a recent study in Pakistan we found that even partial breastfeeding could protect against neonatal sepsis compared to non-breastfeeding with an odds ratio of 18 (*Ashraf* et al., 1990). Only one infant out of the 42 cases of neonatal sepsis and 269 controls was exclusively breast-fed. Comparison showed that partial breastfeeding was much more common among the controls than among the cases ($p<0.001$). Thus partial breastfeeding, protected against neonatal sepsis although the infants had been very early colonised in the gut (*Adlerberth* et al., 1990), presumably via the various fluids and materials given instead of colostrum during the first 1-3 days of life in this region (*Hanson* et al., 1986). It is obvious that the early feeding patterns may play an important role for the outcome of the neonate.

Table 1: Infants not breast-fed (%) in the four population groups in relation to age

Age (months)	1	6	12	18	24
Village	2.4	3.5	4.3	11.1	80.2
(n)	(332)	(289)	(281)	(279)	(262)
Mud hut	5.6	7.4	21.9	50.3	75.4
(n)	(270)	(204)	(196)	(183)	(167)
Urban slum	9.5	27.6	42.9	64.4	87.2
(n)	(264)	(243)	(203)	(177)	(180)
Upper middle class	13.6	59.6	75.2	81.5	95.6
(n)	(162)	(141)	(133)	(130)	(113)

FEEDING PATTERNS OF INFANTS IN PAKISTAN

Whereas breastfeeding with an early onset after delivery is now the rule among Swedish infants, the pattern is different for Pakistani infants. This is obvious from the follow-up of 1473 children in four socio-economically different populations in Pakistan; a village, a mud hut area, a city slum area and an upper middle class control group. First, there was no difference in feeding patterns between boys and girls. Very few infants were exclusively breast-fed, but this number varied with the area of living and with the season of the year (Figure 1). Exclusive breastfeeding was more frequent in the village than in the other three areas. During the hot season April-September exclusive breastfeeding was almost not practised because the mothers gave more extra fluid during this period (*Jalil* et al., 1990). The number of infants given breast milk and water increased strikingly both in the village and the mud hut area during the hot season. The number of only breast-fed decreased simultaneously.

Exclusive as well as partial breastfeeding became continuously less common with age, especially during the second half of the first year. The non breast-fed were more than twice as common in the upper middle class group than in the village or mud hut area during the first 2 years of life (Table 1). During the first month of life the non breast-fed infants were only 2.4% in the village, 5.6% in the mud hut area, 9.5% in the urban slum and 13.6% in the elite group.

Studying the onset of breastfeeding, it was obvious that most started within the first 96 hours of life (*Hanson* et al., 1986). The onset was somewhat earlier in the urban slum and the city elite than in the village and mud hut area where a delay of 48-72 hours was common. Before the onset of breastfeeding the neonates are given other fluids and material including honey, laxatives and herb extracts by spoon, bottle or hand (*Hanson* et al., 1986). The bottle is most common in the upper middle class group, the spoon in the mud hut area and the village, the hand in the urban slum (Table 2). This brings a major risk for exposure to microbial pathogens.

These observations taken together, i.e. the rather late onset of breastfeeding after birth and the rarity of exclusive breastfeeding have consequences for the bacterial colonisation of these new-borns, such as a more hetero-

Table 2: The mode of administration (in %) of extra fluid prior to the onset of breastfeeding in the four population groups

	Spoon	Hand	Bottle	Other
Village	39.5	12.1	5.0	43.4
(n)	(180)	(55)	(23)	(198)
Mud hut	60.3	11.2	13.7	14.8
(n)	(216)	(40)	(49)	(53)
Urban slum	20.3	61.2	4.3	14.2
(66)	(199)	(14)	(46)	
Upper middle class	7.3	14.6	76.4	1.7
(n)	(17)	(34)	(178)	(4)

genous intestinal flora with more potential pathogens, possibly also in higher numbers. This may be one explanation of the high morbidity and mortality of these infants in sepsis/meningitis and early acute and prolonged gastro-enteritis. It is most likely that it would be advantageous to initiate breastfeeding directly after birth and to avoid other often contaminated fluids and foods altogether.

CONCLUSIONS

The foetus responds to microbial antigens, possibly as a result of exposure to transplacental maternal anti-idiotypes against microbial antigens.

Pakistani infants produce higher levels of mucosal IgA antibodies to the intestinal flora during the first weeks of life than Swedish infants. The Pakistani new-borns are also earlier colonised in the gut with Gram-negative aerobes than Swedish infants. The intestinal colonisation of the Pakistani infants is equally early whether they are delivered vaginally or by caesarean section, whether they are delivered at home or in hospital.

The mode of feeding influences the colonisation. Late onset of breast-feeding and giving other foods and fluids than human milk presumably relates to the early intestinal colonisation of the Pakistani infants.

During the hot season of the year the breast-fed babies are given more extra fluid and exclusive breastfeeding becomes non-existent during the period they need the protection of breastfeeding the most.

LITERATURE

Adlerberth, I., Carlsson, B., de Man, P., Jalil, F., Larsson, P., Mellander, L., Svanborg Edén, C., Wold, A., and Hanson, L.Å.: Intestinal colonization with *Enterobacteria* in Pakistani and Swedish Hospital-delivered Infants. Acta Paediatr. Scand. 80, 602-10 (1991).

Andersson, B., Porras, O., Hanson, L.Å., Lagergård, T., and Svanborg Edén, C.: Inhibition of Attachment of *Streptococcus pneumoniae* and *Haemophilus influenzae* by Human Milk and Receptor Oligosaccharides.

J. Infect. Dis. 153, 232-237 (1986).

Ashraf, R.N., Jalil, F., Zaman, S., Karlberg, J., Khan, S.R., Lindblad, B.S., and Hanson, L.Å.: Breastfeeding protects against neonatal sepsis in a high risk population. Arch. Dis. Child. 66, 488-490 (1991).

Goldman, A.S., Thorpe, L.W., Goldblum, R.M., and Hanson, L.Å.: Anti-inflammatory Properties of Human Milk. Acta Paediatr. Scand. 75, 689-695 (1986).

Hahn-Zoric, M., Carlsson, B., Björkander, J., Osterhaus, A.D., Mellander, L., and Hanson, L.Å.: Presence of non-maternal specific antibodies in newborns of mothers with antibody deficiencies. Pediatr. Res. 32, 150-4 (1992).

Hahn-Zoric, M., Carlsson, B., Jeansson, M., Ekre, H.P., Osterhaus, A.D., Roberton, D., and Hanson, L.Å.: Anti-idiotypic antibodies to poliovirus in commercial immunoglobulin preparations, human serum and milk. Pediatr. Res. 33, 475-480 (1993).

Hahn-Zoric, M., Fulconis, F., Minoli, Y., Moro, G., Carlsson, B., Böttiger, M., Räihä, N., and Hanson, L.Å.: Antibody responses to parenteral and oral vaccines are impaired by conventional and low protein formulas as compared to breastfeeding. Acta Paediatr. Scand. 79, 1137-1142 (1990).

Hanson, L.Å., Adlerberth, I., Carlsson, B., Jalil, F., Karlberg, J., Lindblad, B.S., Mellander, L., Khan, S.R., Hasan, R., Sheikh, A.K., and Söderström, T.: Breastfeeding in reality. In: Human lactation 2, maternal and environmental factors (Eds.: Hamosh, M. and Goldman, A.S.). Plenum Press, New York, 1-12 (1986).

Hanson, L.Å., Carlsson, B., Ekre, H.P., Hahn-Zoric, M., Osterhaus, A.D.M.E., and Roberton, D.: Immunoregulation mother-fetus/newborn, a role for anti-idiotypic antibodies? Acta Paediatr. Scand. suppl. 351, 38-41 (1989a).

Hanson, L.Å., Adlerberth, I., Carlsson, B., Dahlgren, U., Hahn-Zoric, M., Jalil, F., Khan, S.R., Larsson, P., Midtvedt, T., Roberton, D., Svanborg Edén, C., and Wold, A.: Colonization with *Enterobacteriacae* and immune response, especially in the neonate. In: The regulatory and protective role of the normal microflora (Eds.: Grubb, R., Midtvedt, T., and Norin, E.). Wenner-Gren Int. Symp. Series, Stockholm Press, New York, 52, 59-69 (1989b).

Jalil, F., Adlerberth, I., Ashraf, R., Carlsson, B., Hanson, L.Å., Karlberg, J., Khalil, K., Lindblad, B.S., Nazir, R., Zaman, S., and Raza Khan, S.: Methodological problems, in assessment of long-term health outcomes in breast-fed versus bottle-fed infants. In: Human lactation 4. Breastfeeding, nutrition, infection and infant growth in developed and emerging countries (Eds.: Atkinson, S., Hanson, L.Å., and Chandra, R.K.). ARTS Biomed. Publ. Distr., St. John's, 381-394 (1990).

Mellander, L., Carlsson, B., and Hanson, L.Å.: Appearance of secretory IgM and IgA antibodies to *Escherichia coli* in saliva during early infancy and childhood. J. Pediatr. 104, 564-568, (1984).

Mellander, L., Carlsson, B., Jalil, F., Söderström, T., and Hanson, L.Å.: Secretory IgA antibody response against *Escherichia coli* antigens in infants in relation to exposure. J. Pediatr. 107, 430-433 (1985).

Mellander, L., Carlsson, B., and Hanson, L.Å.: Secretory IgA and IgM antibodies to *E.coli* O and Poliovirus Type 1 antigens occur in amniotic fluid, meconium and saliva from newborns. A neonatal immune response without antigenic exposure - a result of anti-idiotypic induction? Clin. Exp. Immunol. 63, 555-561 (1986).

Okamoto, Y., Tsutsumi, H., Kumar, N.S., and Ogra, P.L.: Effect of breastfeeding on the development of idiotype antibody response to F glycoprotein of respiratory syncytial virus in infant mice after post-partum maternal immunization. J. Immunol. 142, 2507-2512 (1989).

Stein, K.E. and Söderström, T.: Neonatal administration of idiotype or anti-idiotype primes for protection against *E.coli* K13 infection in mice. J. Exp. Med. 160, 1001-1011 (1984).