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13. POLYSPECIFIC IMMUNOGLOBULINS, THEIR POSSIBLE ROLE IN THE NORMAL (PHYSIOLOGIC) CLEARANCE OF MICRO-ORGANISMS AND TISSUE FRAGMENTS

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THE DEVELOPMENT OF IgM IN EVOLUTION; ITS ROLE IN PRIMITIVE ANIMAL SPECIES

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SUMMARY

The antibodies of immunoglobulin-type appear to be unique defence molecules of jawed vertebrates. Molecules of antibodies are formed by B-lymphocytes, in which the immunoglobulin genes undergo a series of chromosomal rearrangements and somatic mutations, thus generating antibodies with high affinity. This brief review brings in summary form, what is known about defence molecules in deuterostomian echinoderms and invertebrate chordates, and what a role the IgM plays in lower vertebrates.

INTRODUCTION

The defence of integrity of multicellular organisms is realised by means of very different humoral factors comprising a wide collection of molecules ranging from relatively simple lectins and acute phase type proteins which of example could serve C-reactive protein of horseshoe crab, from arthropod antimicrobial factors like attacins, cecropins and many others to oxidative enzymes to highly complex molecules of immunoglobulin superfamily such as specific antibodies and TCR. In *sensu lato*, also recognition molecules, cytokines, clotting factors and various signal molecules could be regarded as defence molecules.

GENERAL REMARKS

Immunoglobulin superfamily

The immunoglobulin multigene superfamily is in actuality an assemblage of smaller subfamilies, all supposingly derived from a common ancestral gene controlling a primordial immunoglobulin molecule, but diverged to separate functions by means of genetic processes.

Main unique features of molecules of immunoglobulin superfamily are multiplicity, close linkage, sequence homology and overlapping functions. Sequence relatedness to other member molecules and presence of ß-sheet globular domain (about 100-110 amino acids in size to either constant or variable domains of an immunoglobulin molecule) are main prerequisites for a molecule to be admitted to membership in the immunoglobulin superfamily (*Williams* and *Barclay* 1988).

Evolution of immunoglobulins

It is likely that molecules of immunoglobulin superfamily emerged from cell adhesion molecules (CAM) of plasma membranes known as cadherins

that first evolved to mediate interactions between cells (*Ohno*, 1996). They have been found in protostome invertebrates where they are more related to neural cell adhesion molecules (N-CAM) than immunoglobulins (*Hughes*, 1998).

The unequal crossing over, gene conversions and the residence on different chromosomes may be the primary force driving the evolution of the Ig superfamily. Possible mechanism is gene duplication from a primordial gene coding for about 100 amino acids forming a single Ig-domain. There are two forms of immunoglobulin molecule, the soluble antibody and the membrane-bound B-cell receptor for antigen which of immunoglobulin heavy chain C-terminal is encoded by a single gene.

It seems more probable that the emergence of the primordial immunoglobulin domain and its consequent duplication and diversification into L- and H-chains, and T-cell receptor domains had to occur within a relative short time span during evolution of gnathostomian ancestors, probably 10 million years (*Marchalonis* et al., 1998). It was proposed that the horizontal transfer of originally microbial genes RAG (recombination activating genes) and their incorporation into genomes of predecessors of jawed vertebrates could be an important evolutionary event which caused the duplication and rearrangement of the ancestral immunoglobulin gene elements, the capability of which could be utilised for the recognition of alien and the response to it (*Bernstein* et al., 1996b). This event can be considered, similarly to cosmologic hypothesis, as a "big-bang" for the onset of vertebrate adaptive immunity. The second key evolutionary event was invention to arrange gene segments V, D, J, and C.

From the evolutionary point of view, a candidate for primordial immunoglobulin molecule could serve $ß_2$-microglobulin consisted of 99 amino acid residues in single chain with one intrachain disulphide bond. It is ubiquitous on all mammalian cells with exception of red blood cells and a molecule with considerably high homology has been also found in many invertebrates. It was suggested that ancestral $ß_2$-microglobulin gene could diversified into a "primitive gene" in protostomes and into "primordial gene" in deuterostomes. From deuterostomian precursor, $ß_2$-microglobulin genes and immunoglobulin genes arose by rapid evolutionary diversification (*Shalev* et al., 1983). Similarly, the Thy-1 molecule is more primitive than immunoglobulin and MHC molecules. For the Thy-1 homologue was found in annelids, molluscs, and tunicates, it could be supposed that the Thy-1 gene may be evolutionary very closely related to the primordial gene for immunoglobulin and MHC in vertebrates (*Stewart*, 1992).

Generation of antibodies

The presence of multiple V genes in the genome is a fundamental *condicio sine qua non* for generation of antibody diversity. It is assumed that the evolution of immunoglobulin H-chain V region could last for 150-200 million years. Ontogenetic generation of the antibody repertoire is dependent on germline encoded V genes and D and J segments. Mammals generate antibody repertoire by many hundreds of V genes and several D and J segments (*Tonegawa*, 1983). In birds, the antibodies emerge by diversifying one or limited number of V genes and many pseudo-V genes through the process of gene conversion (*Reynaud* et al., 1985). The antibody repertoire of anuran amphibians seems to be rather re-

Table 1: The most important defence molecules of echinoderms

INVERTEBRATE TYPE FACTORS
(haem)agglutinins(C-type lectins), **(haemo)lysins, perforins,** (proteins)
involved in: agglutination, cell adhesion, cell lysis

FACTORS UNIQUE FOR ECHINODERMS
sea star factor, profilin (proteins)
involved in: inflammation, inhibition of macrophage, suppression of T-dependent mammalian immune response, signal transduction
antibody-like protein
involved in: inducible, complement-dependent lysis?

VERTEBRATE-LIKE FACTORS
IL-1-like, IL-2-like, IL-6-like, TNF-like, IFN-γR (proteins)
involved in: stimulation of proteosynthesis and phagocytosis, inflammation, cytotoxicity
C3-like (homologue to a vertebrate complement component)
involved in: opsonin ? primitive alternative pathway?

MEMBERS OF IG SUPERFAMILY
IL-1R, IL-6R (receptors)
involved in: ?

stricted but their V genes have been shown to have tendency to hypermutate (*Wilson* et al., 1992). The fish antibody repertoire is also limited and the affinity maturation during the humoral immune response is not fully manifested (*Witzel* and *Charlemagne*, 1985).

THE VECTORS OF HUMORAL IMMUNITY OF DEUTEROSTOMIAN INVERTEBRATES

The echinoderms

The members of phylum *Echinodermata* are thought to have originated from a common ancestor with chordates. Although their larvae are bilaterally symmetrical, they gain the secondary pentameric radial arrangement of their bodies.

Immunocompetent structures

The echinoderms are not endowed by any structures or differentiated immunocompetent organs. Phagocytic sessile and dispersed coelomocytes could be analogised as the vertebrate "reticuloendothelial system." The axial organ has similar poietic and phagocytic functions to the spleen and lymph node, respectively. Its cells are differentiated into phagocytic and lymphoid-like cells being divided into B-like and T-like cells.

Defence molecules

Several interesting humoral factors playing a role in echinoderm humoral defence have been described, namely (haem)agglutinins (lectins), complement-like and lysozyme-like molecules (Table 1). Some molecules act as perforins or in signal transmission like profilins (for review see *Matranaga*, 1996).

Sea star factor of *Asteria forbesi* (*Prendergast* and *Suzuki*, 1970) has significant suppressive effects on the development of T-dependent antibody secreting clones by preventing lymphokine secretion (*Kerlin* et al., 1994).

In *A. forbesi*, the presence of regulatory cytokine-like molecules, an IL-1-like and IL-6-like proteins with conserved amino acid sequence when compared to mammalian interleukine counterparts, was described (*Beck* and *Habicht*, 1996). In addition, cytokines released by axial organ T-like cells with properties similar to vertebrate IL-1 and IL-2 (*Leclerc*, 1996) and cell surface structures resembling human receptors for TNF, IFN-γ, IL-1, and IL-6, were found in *A. rubens*.

The axial organ B-like cells of sea star, in many aspects similar to vertebrate lymphocytes, were found to react to the antigenic challenge by the secretion of a specific antibody-like protein capable of lysis of haptenated erythrocytes in the presence of complement (*Delmotte* et al., 1986). Compared to the immunoglobulin of lower vertebrates, this antibody-like substance is structurally simpler, not having L- and H-chains.

On the basis of molecular analyses, a primitive homologue of complement system composed of at least one component (C3) and a receptor was identified in sea urchin *Strongylocentrotus purpuratus*. This system might serve as a platform onto which the Ig gene system was latter added and resulted in the sudden expansion of both systems in higher vertebrates (*Smith* et al., 1996).

In conclusion, echinoderms like other invertebrates secrete various non-specific humoral immune substances into their coelomic fluid. Despite of this fact, the echinoderms have developed an efficient immune strategy with some interesting similarities in fundamental defence features to the vertebrates. The presence of receptors for IL-1 and IL-6, the members of Ig superfamily, and cytokine-like factors such as IL-1, IL-2, IL-6 or TNF provides support for a continuity of rearranging Ig molecules and immunoregulative molecules in at least deuterostome phylogeny (*Legac* et al., 1996).

THE VECTORS OF HUMORAL IMMUNITY OF INVERTEBRATE CHORDATES

The tunicates

The tunicates are considered to be the most primitive present-living members of phylum *Chordata*. Owing to their evolutionary position they have attained attention of comparative immunologists as organisms in which first traces of adaptive immunity could be found.

Immunocompetent structures

First distinct mesodermal-derived haemopoietic structures have developed in ascidians. Accumulations of stem haemoblasts are located in mesenchymal tissues, diffused or structured into “lymph nodules“ along the digestive tract. Structural composition of pharyngeal wall, where mutual interactions among ectodermal epithelium, mesenchymal tissue, and endoderm took place, could serve as a background for later developmental potential for the thymic emergence in vertebrates. The mesenchymal origin of blood cells, of which one type is almost indistiguishable from vertebrate lymphoid cells and the organisation of nodules in the tight vicinity with the pharyngeal region and the gut, are common features shared with all vertebrates.

Defence molecules

Similar to protostomian invertebrates, substances involved in immune system of tunicates are prevailably haemagglutinins (lectins, some of them

Table 2: The most important defence molecules of tunicates

INVERTEBRATE TYPE FACTORS

(haem)agglutinins C-type lectins), opsonins (various)

involved in: agglutination, cell adhesion and migration, LPS-binding, opsonisation, stimulation of cell proliferation, recognition, phagocytosis

FACTORS UNIQUE FOR TUNICATES

lamellarins (poly-aromatic alkaloids), **crucigasterins** (poly-unsaturated amino-alcohols), **dicyclamids, didemnins, ulithiacyclamids, patellamids** (cyclic peptides), **halocyamins** (dihydrotryptamins), **substance Ete** (cytokine-like), **ecteinascidins,** eudistomins (β-carboline derivatives)

involved in: antiviral, antimicrobial and antineoplastic activities, cytotoxicity, stimulation of phagocytosis

complement control superfamily factors

involved in: ?

VERTEBRATE-LIKE FACTORS

IL-1α, IL-1β, (C-type lectins)

involved in: stimulation of cell proliferation. co-mitogenic

MEMBERS OF IG SUPERFAMILY

Thy-1 homologue, Lyt-2/3 (CD8) homologue

involved in: ?

even more similar to plant lectins rather than animal lectins), various antimicrobial and antiviral substances, opsonins, enzymes, poly-aromatic alkaloids or cyclic peptides, and cytokine-like substances (Table 2). Cytokine-like protein with the similar activity to IL-1 was found in a row of tunicate species (*Beck* et al., 1989; *Raftos* et al., 1992). The opsonin isolated from *Styella clava* is functionally and physicochemically similar to acute phase proteins of mammals (*Kelly* et al., 1992). Analyses of urochordate cDNA revealed a 50% identity with the short consensus repeats of the human complement factors, most of all factor H, apolipoprotein H, or complement receptors type 1 and 2 (*Pancer* et al., 1995). Hypothetically, this polymorph sequence might represent and ancestral molecular prototype in evolution of complement control protein superfamily.

In 1968 F.M. Burnet suggested that haemagglutinins documented in tunicates might be forerunners of vertebrate immunoglobulins. It has been shown by *Rosenhein* et al., (1985) that some components of the plasma of *Halocynthia pyriformis* and *Boltenia ovifera* react with antisera against shark immunoglobulin 7S heavy chain. Therefore, the possibility cannot be ruled out that these plasmatic proteins could represent the ancestral precursor of immunoglobulin molecule. In 1984 a molecule cross-reactive with the shark μ-chain (μCRM) was isolated from the haemolymph of *Pyura hausteria* and *B.ovifera*. On the basis of immunochemical data and sequence homologies, the relationship of tunicate μCRM to Ig is indicated (*Schluter* et al., 1994). The occurrence of a Thy-1 homologue, a simple member of immunoglobulin superfamily, was ascertained in a solitary tunicate *S. clava* (*Mansour* and *Cooper*, 1984). Testing of tunicate haemocytes for the possible expression of Lyt antigens revealed a homologue of the murine Lyt2/3 complex (CD8), another molecular member of immunoglobulin superfamily (*Negm* et al., 1992).

Table. 3: The most important defence molecules of cyclostomes

INVERTEBRATE TYPE FACTORS
haemagglutinins (C-type lectins), **opsonins, bactericidins** (various), **haemolysins** (proteins)
involved in: agglutination, cell adhesion, antimicrobial, cell lysis

FACTORS UNIQUE FOR CYCLOSTOMES
?

VERTEBRATE-LIKE FACTORS
C3, C4, C5 (complement components)
involved in: alternative pathway, devoid of lytic action

MEMBERS OF IG SUPERFAMILY
?

Conclusively, the tunicates have developed immune phenomena like inflammation, genetically controlled non-fusion (colony-specific) reaction or allogeneic recognition and rejection of tissue grafts, which strongly resemble those of vertebrates. Although tunicates do not have some any antibodies or complement, the presence of cytokine-like substances suggests a possible common ancestry of some constitutive defence substances in advanced vertebrates with defence factors of invertebrate chordates or even invertebrates. The possibility cannot be ruled out that some of these molecules are structurally very close to primordial immunoglobulin molecule. The presence of Thy-1 and Lyt2/3 homologues, the members of Ig superfamily, documents tunicate key position in evolutionary pathways to vertebrate immunoglobulins.

THE VECTORS OF HUMORAL IMMUNITY OF JAWLESS VERTEBRATES

The cyclostomes

The members of extant two orders of cyclostomes, hagfish and lampreys, represent the most primitive species of the vertebrates. Comparative immunologists do not often consider the fact that the myxinids profoundly differ from petromyzontids in many embryological, anatomical, physiological and biochemical features. The lampreys could be categorised as a sister group of jawed vertebrates, whereas the hagfish have evolved independently.

Lymphoid tissues and organs

Equivalents of bone marrow, spleen, and lymph nodes may be preferentially attributed to pronephros and supraneural body, typhlosole, and cavernous body, but the distinct diversification onto primary (thymus, Bursa of Fabricius) and secondary immune organs and tissues seems to be absent (*Zapata* and *Cooper*, 1990).

Defence molecules

Main vectors of humoral immunity in cyclostomes are naturally occurring haemagglutinins and haemolysins and proteins homologous to the C3, C4, and C5 vertebrate complement components (Table 3). Cyclostome complement resembles an alternative pathway. It is devoid of significant lytic action, but

acts as the essential phagocytic factor (*Nonaka* and *Takahashi*, 1992; *Fujii* et al., 1995). Although some authors (e.g. *Varner* et al., 1991) had reported sequence similarities between a hagfish protein believed to be an immunoglobulin, molecular sequence analyses clearly proved it was a serum complement protein (*Ishiguro* et al., 1992). Moreover, the presence of genes for TCR, MHC and RAG in either hagfish or lampreys has not been described (*Klein* and *Sato*, 1998).

Mucosal immunity

Ancient agnathans were probably microphagous animals. Present-living cyclostomes are secondary specialised to parasitic life. Primitive foci of lymphoid cells (e.g. in branchial region specialised in trapping of particles) and lymphohaemopoietic aggregations accompanying veins of the gut (e.g. in the intestine submucosa of hagfish and typhlosole of lampreys) function analogically as GALT of endotherms (*Tanaka* et al., 1981). Nevertheless, neutrophil leukocytes form the most abundant cell population of the hagfish intestine. In lampreys, the plasmacytes with conspicuous morphology were distinguished in the typhlosole. It seems more probable that the lymphomyeloid intestinal tissue has poietic rather than immune functions thus resembling bone marrow.

Conclusively, both hagfish and lampreys lack major immune tissues and organs like thymus, spleen, and true hierarchised GALT. It is not definitively known, if the T and B cell dichotomy exists. Moreover, the absence of immunoglobulins implies that the present-living species of cyclostomes are endowed by the invertebrate type of constitutive immunity. From comparative immunology standpoint, and contrary to previous opinions, cyclostomes are clearly demarcated from other vertebrates.

THE IMMUNOGLOBULINS OF JAWED VERTEBRATES

The cartilaginous fish

The members of cartilaginous fish are divided into two main assemblages, the elasmobranchs comprising modern sharks, skates and rays, and the holocephalans, the chimeras. Studies of immunity have focused on mainly carcharhine sharks which arose approximately 60 million years ago, and in present forming more than 50% of all extant elasmobranchs, some representatives of related squalomorphs, more distant and older groups of heterodontid sharks, rays and chimeras.

Lymphoid tissues and organs

The thymus is multilobulated organ clearly consisting of cortex and medulla. The spleen has been proved to be major organ of the antibody formation. Lymphomyeloid structures, which may substitute the bone marrow and lymph nodes, such as organ of Leydig or epigonal organs are located in the oesophagus and in the gonads, respectively. Lymphocytes, plasmacytes, and macrophages, similar to those of all jawed vertebrates, have been described in these animals (*Zapata* et al., 1996).

Immune molecules of immunoglobuliny superfamily

All representatives of cartilaginous fish studied up to the present possess genes for expression of immunoglobulins, T-cell receptors (α/β and γ/δ), MHC class I and II, and RAG-1 (*Marchalonis* et al., 1998) (Table 4).

Table 4: The most important defence molecules of cartilaginous fish

NATURAL FACTORS

(haem)agglutinins (C-type lectins), **(haemo)lysins** (lytic enzymes), **acute phase proteins, complement** (both pathways), **squalamine** (aminosterol antibiotic)

involved in: agglutination and lysis, opsonisation, regeneration and wound healing, phagocytosis and chemotaxis, inflammation, antibacterial and antifungal activity

CYTOKINES

?

IMMUNOGLOBULIN SUPERFAMILY

IgM, (pentamer, dimer and monomer molecules), **IgNARC** (chimeric molecule), **NAR, IgW** (orthologue of IgNARC), **IgR (IgX), TCRα/β TCRγ/δ, MHC class I, class II, β2-microglobulin**

involved in: humoral immunity (mucous and serum antibodies), recognition, transplantation immunity

Immunoglobulins

Cartilaginous fish respond to antigen challenge by producing of elevated levels of specific antibodies. Secondary and repeated immunisations do not increase either quantity or the affinity of antibodies. It may be consistent with the restriction of antibodies to the IgM isotype and also with the unique arrangement of immunoglobulin gene segments. IgM molecule is a pentamer (17S -19S) of the basic structure H_2L_2 and a H-chain composed of four C domains. In the serum of some shark species, monomers and dimers of IgM have been found. For a survey of older studies of shark immunoglobulins see *Vêtviçka* and *Síma* (1998). In 1996, two new non-IgM isotypes were reported in nurse shark, IgNAR and IgNARC (Ig new antigen receptor from cartilaginous fish). The IgNARC appears to correspond to IgW of sandbar shark (*Bernstein* et al., 1996a; *Greenberg* et al., 1996). The IgNAR molecule is a dimer consisting of a modified ω H-chains comprising one V and five C domains but no L-chains. The IgW molecule overall resembling μ chains of IgR (IgX), another isotype described in skates and rays and mammalian IgM, is considered to be a surface molecule of shark B cell. Unlike any other H-chain-type molecule, it is comprised of six C domains. It is suggested that IgW may retain ancestral features of immunoglobulin molecule.

In contrary to mammals, chondrichthyans possess two distinct sets of H-chain V regions: Vω, which occur only in sharks and may be in other species of cartilaginous fish, and the V region associated with μ chains which could be a derivative of common ancestor shared with V_H domains of all jawed vertebrates. The shark Vμ shares greater identity to mammalian counterpart than it does Vω taken from the same species. The evolutionary separation of constant domains Cω and Cμ had also to be a very ancient event in the history of cartilaginous fish because they share very low identities when compared among different species. A different situation exists in skates where an 18S IgM molecule and 8.9S IgR molecule have been described. The IgR is produced by the plasma cells in a different way from those secreting IgM (*Tomonaga* et al., 1984).

V, D, J, and C gene segments occur in individual clusters apparently unlinked to one another, rather than in the translocon arrangements typical for

mammals (*Hinds* and *Litman*, 1986). The sharks, rays, and skates are unique in having distinctive multicluster or multiple locus arrangement for the H-chain of their IgM and IgR, in which units of V_H-D_H-D_H-J_H-C_H are many times (approximately 100 times) repeated on the chromosome. L-chain genes show the similar type of repeated multicluster gene organisation with V_L-J_L -C_L. Sharks possess three classes of L-chains: κ, λ and a type restricted to them. About at least three distinct families of λ chains are organised into 200 separate clusters of V_L segments, each of which contains an individual V, J and C segment (*Shan* et al., 1996). This configuration of the IgH and the IgL loci is possible reason for the restriction of combinational diversity, even though the number of clusters may be very large. Restriction of antibody diversity is caused by joining of V_H-D-J_H segments in the germ-line and by unique type of rearrangement which takes place within a cluster but not between clusters. The loci for IgNAR and IgNARC are small with a few genes arranged in clusters. Up to date there is no information on the IgW locus (for rev. see *Warr*, 1995).

Mucosal immunity - the jaw hypothesis

"The new ability to bite and swallow food by animals with the jaw would have caused increased frequency of physical injuries in the wall of digestive tract (oesophagus, stomach and intestine) of those primitive jawed fishes, which eventually led to the development of adaptive immunity." (*Matsunaga* and *Andersson*, 1994; *Andersson* and *Matsunaga*, 1996). It could be easily imagined that these animals explored all available mechanisms which they had inherited from their ancestors for watching and defending their digestive tract. Thus, it is understandable that from the first jawed animals, the GALT has gained its main immune importance. In contrast to cyclostomes, true small lymphoid nodule-like or massive accumulations of granular, macrophage, lymphoid, and plasma cells including antibody forming cells can be seen in a spiral valve or duodenal lamina propria and gut epithelia. IgM has been detected in the gut mucus and bile in quantities similar to serum levels (*Hart* et al., 1987).

Generally, the cartilaginous fish are the first vertebrates exhibiting true adaptive immunity. For the first time in the phylogeny of vertebrates, the major immunocompetent tissues are structuralised as distinct organs. On the other hand, neither true effector functions of T lymphocytes nor T and B co-operation have been described. The study of cytokines have, thus far, been omitted in chondrichthyans. Surprisingly, the IgM is not the primordial immunoglobulin isotype and the arsenal of immunoglobulin classes is greater than previously supposed. Nevertheless, there is a lack of affinity maturation of antibodies during the immune response.

The bony fish

Because of being under the continuous selective pressure of aqueous environment, the fish retained a high degree of conservatism in their body plan which is shared by all fish taxa independently of their kinship. That is the reason why the seemingly compact taxa of fish are more heterogeneous and phylogenetical distances among them are much more greater than among the mammals. A very limited sample of fish has been studied from the point of their immunity. Substantial knowledge is available only on fishes of commercial importance, whereas the deep-sea or pelagic fishes are omitted. Despite these limitations we are more or less able to deduce the general pattern of their immune capacities.

Table 5: The most important defence molecules of bony fish

NATURAL FACTORS

(haem)agglutinins (C-type lectins), **(haemo)lysins** (lytic enzymes), **opsonins** (various), **granulins** (epithelin family growth factors), **lipoxins** (eicosanoids), **C-reactive protein, amyloid P** (pentraxins, acute phase proteins), **complement** (both pathways)

involved in: agglutination and lysis, opsonisation, regeneration and wound healing, phagocytosis and chemotaxis, inflammation

CYTOKINES

CSF, IL-1α/β, IL-2-like, IFN-like (IFN-α?, IFN-β), TGF-β, TNF-α

involved in: immune reactions (antiviral), immune processes regulation, growth factors, cell proliferation stimulators

IMMUNOGLOBULIN SUPERFAMILY

IgM (tetramer and monomer molecules), **IgN**, **IgD-like?** (chimeric molecule), **TCRα/β, TCRγ/δ, MHC class I**, **class II, β2-microglobulin**

involved in: humoral immunity (mucous and serum antibodies), recognition, transplantation immunity

Lymphoid tissues and organs

Like in chondrichthyans, the thymus and spleen are fish major lymphoid organs. The thymus cortex and medulla are lacking in most species. There is only limited knowledge on the maturation of thymocytes and the origin of T-cell specificity in immune response. For the first time in the phylogeny, the structures similar to Hassall's corpuscles have been reported in some species. The fish spleen is a main site where formation of antibodies takes place. The absence of germinal centres is compensated by melano-macrophage centres and ellipsoid sheets. The kidney and particularly the well-developed GALT serve as analogues of bone marrow and lymph nodes. They represent major sites of haemopoiesis together with processing of antigens, and production of antibodies. The B and T cell dichotomy in fish has been documented by means of monoclonal antibodies (*Koumans-van Diepen* et al., 1995; *Rombout* et al., 1997).

Immune molecules of immunoglobulin superfamily

As in cartilaginous fish, in all species of bony fish studied, the genes for immunoglobulin, TCRα/β TCRγ/δ, β2-microglobulin, MHC I and MHC II, and RAG-1 were documented (*Matsunaga* and *Rahman*, 1998). It was suggested that fish TCR may be close in shape to the ancestral molecule (for review see *Press*, 1998) (Table 5).

Immunoglobulins

Bony fish, unlike cartilaginous fish, synthesise only IgM class antibody, which is tetrameric (in contrast to the pentameric structure of other gnathostomes) without J-chain. Numerous studies have confirmed the fish H-chain is rearranged from V_H, D_H, J_H and C_H genes, and organisation of these genes is variable (*Warr*, 1995). The genomic organisation of IgH-chain locus in fish is of "translocon type" found in amphibians and mammals, as V_H, D_H, and J_H exons are located in separate regions undergoing somatic reorganisation and splicing with a single C_H element (*Wilson* et al., 1995). Like in mammals possessing more than 100 V_H genes grouped in 7 families in the human and 14 in the mouse, from at least 2 to 11 V_H families, in which the number of V_H

genes is well over 100, were identified in teleost fish studied in this respect. Fish L-chains differ from H-chains in having only single C region domain in addition to the V domain. Gene segments for L-chains show the multicluster type of organisation, with V_L, J_L and C_L segment represented in each cluster, D region is absent. Fish L-chain population is heterologous, the L-chains fall into a number of distinct structural types. Whether of not fish L-chain types could be analogised to mammalian κ and λ chains is to be verified. A novel chimeric IgH chain, partly homologous to IgG was reported recently (*Wilson* et al., 1997). It contains a rearranged V domain, the first C domain of μ, and seven C domains encoded by δ gene homologue. On the basis of these analyses, it could be hypothesised that the IgD was primarily an ancient immunoglobulin molecule present in predecessors of both mammals and bony fishes. To be able to make any conclusion about this surprising finding, one should wait for an independent confirmation. Some species of fish possess low-molecular weight immunoglobulins, a monomeric form of IgM (*Wilson* and *Warr*, 1992), a smaller form of monomeric IgM designated IgM(ΔFc) (*Clem*, 1971), and IgN in the lungfish (*Marchalonis*, 1969).

Mucosal immunity of fish

Fish are the first vertebrates in which specific secretory immunity was observed. The skin mucus forms an important barrier in prevention of penetration of pathogenic bacteria and fungi. Besides immunoglobulins, the non-specific factors like (haemo)lysins, (haem)agglutinins (lectins), lytic enzymes, lysozyme, C-reactive protein, and complement are also present.

In some species, intraperitoneal injection of various antigens results in the formation of specific antibodies not only in the blood serum, but also in skin mucus and in the lamina propria of the gut (for review see *Kaatari* and *Piganelli*, 1996). In most studied species of fish, whole complex of GALT functionally approximates the intestine barrier of mammals. The intestinal wall is infiltrated by lymphocytes, granulocytes and other types of cells often aggregated in the mucosa and lamina propria, even if the structures resembling typical Peyer's patches are lacking. Similarly, lymphocytes secreting immunoglobulins occur in skin and gills. In chondrosteans (paddle fish, sturgeon) and in sarcopterygian lungfish, the spiral valve still develops and it is similarly infiltrated by lymphoid cells, often in aggregates, as in cartilaginous fish. In more advanced teleosts, scattered lymphocytes, plasmacytes granulocytes and macrophages, and their aggregates occur in and under the intestinal epithelium particularly in the posterior part of the gut (hind gut). The epithelial cells of the gut may play the same role in food antigen collecting as M cells of Peyer's patches. In the seahorse, the secondary specialised microphagous fish, which has altered the mouth structure and feeding habits, no lymphoid cells or lymphoid cell aggregates in the lamina propria were found, while they are retained in kidney or other locations of their body (*Matsunaga* and *Rahman*, 1998).

In summary, the present-living species of bony fish have developed proper modification of adaptive immunity during million years of their divergence from hypothetical common gnathostome ancestors. The organisation of their lymphoid tissue represents a further advance. The bony fish have retained some fundamental immune mechanisms from the epoch of their ancestral origin, from which as an example could serve preponderant tetrameric IgM molecule. On the other hand, the presence of regulatory cytokines, the

IFN-γ, Il-1 and IL-2-like factors, and macrophage activating factor (for review see *Vêtviçka* and *Síma*, 1998) could suggest that fish cellular and humoral immune processes tightly approximate to those of more advanced tetrapods.

CONCLUSION

The vertebrate immune system did not spring out of nothing. The ancestral molecules which gained later in phylogeny the function of specific immune humoral factors had to be already present in invertebrate animals forming a progenitory assemblage from which the predecessors of deuterostomian chordates have evolved. It might be namely the primitive immunoglobulin molecule, which in echinoderms, invertebrate chordates, and vertebrate cyclostomates did not developed due to a lack of a structuralised lymphoid tissue providing the suitable internal micro-environment for the induction of molecular variability. This morpho-functional background for these events started to develop together with the emergence of jaws and the fundamental body plan of gnathostomean vertebrates. Obviously, the adaptive immune system is an invention of jawed vertebrates. On this stage of evolution, first true immunoglobulin molecules appears: NAR, IgNARC, IgW, IgR, and IgM. Only the IgM seems to become the universal class of immunoglobulins for all advanced vertebrates.

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MATERNAL IMMUNOLOGICAL EXPERIENCE GUIDES THE EDUCATION OF THE NEONATAL IMMUNE SYSTEM

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SUMMARY

Immune responses to thymus-dependent antigens undergo immune maturation through somatic hypermutations which accumulate with repeated immunisations and comprise the memory B cell as well as the memory antibody pool. Consequently, it can be concluded that the B lymphocyte compartment, as a whole, stepwise acquires a knowledge of the external antigenic world with increasing quality and precision. This progression may be regarded as an ontogenetic learning process the development of which, although strictly based on and starting from a genetic basis, is *entirely* dependent on the interaction with the immunologically relevant environment and is only driven by adequate stimuli. Since early ontogeny is an exceptionally sensitive phase for the development of the immune system, we asked whether the immunological experience of the mother, represented by IgG antibodies from different stages of immune maturation, might influence a particular immune response in the offspring. The following results were obtained: 1. An immunisation of female CBA mice with phospholipase A_2 (the main allergen of bee venom) as well as the transfer to the offspring of a mixture or one single monoclonal anti-PLA_2 antibody suppressed the IgE immune responsiveness to PLA_2 in F1 animals for a long period of time. 2. The maternal influence on the quality of immune response was tested in the well explored response to the hapten 2-phenyloxazolone (phOx) in BALB/c mice. BALB/c dams received a primary, secondary or tertiary immunisation with phOx-coupled chicken serum albumin and the primary anti-phOx response was investigated in offspring of the F1 or F2 generation at an age when no maternally derived anti-phOx serum antibodies could be detected. (a) The kinetics of the primary response were altered. (b) About half of the F2 mice developed maximal antibody levels as in a secondary response. (c) The expression of the normally dominant Ox1 idiotype (Id_{Ox1}) was rendered exceedingly variable. (d) Half of the non-Id_{Ox1} anti-phOx antibodies exhibited strongly enhanced affinities, being either identical to or even 7-25 times higher than those of Id_{Ox1} antibodies which are normally of highest affinity. Hence, maternal antibodies of the late stages of immune maturation, which may be perceived as acquired immunological phenotypic characters, have the capacity to interfere with isotype regulation and improve the quality of a considerable proportion of antigen-reactive antibodies in the available repertoire and thus are suspected to give the offspring a benefit in handling of external antigens.

INTRODUCTION

The immune system is endowed with the capacity to develop a state of memory of former events. This memory is only induced in cells which generate adaptive immune responses, namely T- and B lymphocytes. In contrast, those cells which are responsible for the first line of defence and form the innate immune system, i.e. granulocytes and monocytes/macrophages, are not able to develop memory. The formation of immunological memory depends on the activation of T lymphocytes and thus can only be induced by thymus-dependent (TD) antigens. Consequently, memory itself is thymus-dependent. However, whereas antigen recognition of T memory cells is of the same quality as that of virgin T cells, can B cells develop an improved quality = affinity of their antigen receptors during the primary and the following antigen-induced immune responses. This is demonstrated by the fact that TD antibody responses show immune maturation (*Eisen* and *Siskind*, 1964) which is brought about by somatic hypermutations (*Rodwell* et al., 1983; *Griffiths* et al., 1984; *Wysocki* et al., 1986; *Cumano* and *Rajewsky*, 1986; *Berek* and *Milstein*, 1987). Hence, mutated antibodies may be perceived as acquired immunological phenotypic characters. Since these somatic mutations accumulate with repeated immunisations and comprise the memory B cell pool (*Wysocki* et al, 1986; *Berek* and *Milstein*, 1987; *Weiss* and *Rajewsky*, 1990), it can be concluded that the B lymphocyte compartment, as a whole, stepwise acquires a knowledge of the external antigenic world with increasing quality and precision. Hence, this progression can be regarded as an ontogenetic learning process the development of which, although strictly based on and starting from a genetic basis, is *entirely* dependent on the interaction with the immunologically relevant environment and is only driven by adequate stimuli.

The induction of an immune response with a thymus-dependent antigen, e.g. with proteins from normal commensal or pathogenic microorganisms, activates B lymphocytes which, after differentiation to plasma cells, secrete large amounts of immunoglobulins. Their variable domains of the H and the L chain harbour individual antigenic determinants termed *idiotopes* which are not only immunogenic in the xenogeneic and allogeneic, but also in the syngeneic and the autologous host. A particular antibody expresses in its V regions a collection of idiotopes which form the idiotype (Id). The antigen-induced increased synthesis of antibodies (= Id = Ab1) can lead to activation or suppression of B cells expressing antigen receptors reacting with idiotopes of Ab1. Those antibodies activated in the second step are designated anti-idiotypic (aId) or Ab2 and their variable region idiotopes are recognised again by a third set of B cell receptors, named anti-(anti-idiotypic) or Ab3 and so forth. Each of these activation steps leads to a quite heterogeneous population of antibodies. This holds true for the Id population which is activated by antigen as well as for the populations of aId (Ab2) and anti-(anti-Id) (Ab3). The extrapolation of this chain of reactions led to the formulation of the idiotypic network theory (*Jerne*, 1974, 1985; *Jerne* et al., 1982).

Although interactions between an idiotype with its anti-idiotype may, depending on the immunising dose, result in stimulation or suppression of the reactive partner (*Takemori* and *Rajewsky*, 1984a, 1984b), the suppressive effects of anti-idiotypic antibodies have been in the foreground of investigations since they were regarded as important regulatory compounds to prevent overshooting of immune responses. While the

suppression of a particular idiotype by anti-idiotypic antibodies in adult animals is transient in nature, it may especially be long-lasting when the anti-idiotypic response is induced in new-borns shortly after birth (*Strayer* et al, 1975; *Augustin* and *Cosenza*, 1976; *Hiernaux* et al., 1981) or the corresponding idiotype may even be permanently lost (*Kearney* et al., 1983; *Vakil* et al., 1986). When anti-idiotypic antibodies are actively induced or injected into pregnant mothers and reach the foetus via the maternal route before and/or after birth, they also suppress the corresponding idiotype in the offspring (*Weiler* et al., 1977; *Victor* et al., 1983). Interestingly, if an anti-idiotypic manipulation either by direct immunisation of the neonate or via the mother is directed towards a highly connected idiotype expressed by multispecific, cross-reactive IgM antibodies, a long-term or permanent severe disturbance of a larger part or even the *entire* antibody repertoire of that animal may occur (*Vakil* et al., 1986; *Bernabe* et al., 1981). Moreover, it has been shown that the transfer of idiotypes or anti-idiotypes solely with the colostrum and milk after birth is sufficient to induce idiotypic interaction and / or protection against microbial infection from the mother to the offspring (*Weiler* et al., 1977; *Rothstein* and *Vastola*, 1984; *Stein*, 1985; *Ali* et al., 1988; *Heiman* and *Weisman*, 1989). This clearly emphasises the importance of natural postnatal rearing and supports the idea that not only the experimentally induced but also naturally-occurring antibodies of the mother can influence the development of the new-borns´ immune system and the generation of the antibody repertoire (*Wikler* et al., 1980; *Bernabe* et al., 1981; *Stein*, 1985; *Kearney* et al., 1986; *Martinez* et al., 1986; *Vakil* et al., 1986; *Andrade* et al., 1990).

Based on such idiotypic interactions in the immune system, we assumed: (a) Highly maturated antibodies which are transferred before and/or after birth from the dams to the offspring will not only confer passive protection, but are expected to influence a particular immune response in the offspring, probably induced by idiotypic-anti-idiotypic interactions. (b) Furthermore, maternal antibodies will supposedly not only induce a clonal alteration of unknown functional relevance for an antigen-induced immune response, but might influence the quality of immune responses in the offspring in a biologically meaningful fashion. These assumptions were tested by determining the IgE-regulatory potency of maternally derived monoclonal IgG antibodies and by analysing the primary immune response to the hapten 2-phenyl-oxazolone in F1 and F2 offspring of dams which had been immunised or injected with anti-phOx monoclonal antibodies.

METHODS

Animals

The immune response to 2-phenyl-oxazolone (phOx) was studied in BALB/c (H-2^d) mice and that to phospholipase A_2 (PLA_2) in CBA/J (H-2^k) mice, both of which were obtained from Harlan/Winkelmann (Borken, Germany) or Bomholtgaard (Ry, Denmark) and reared under conventional conditions in the animal house of the university.

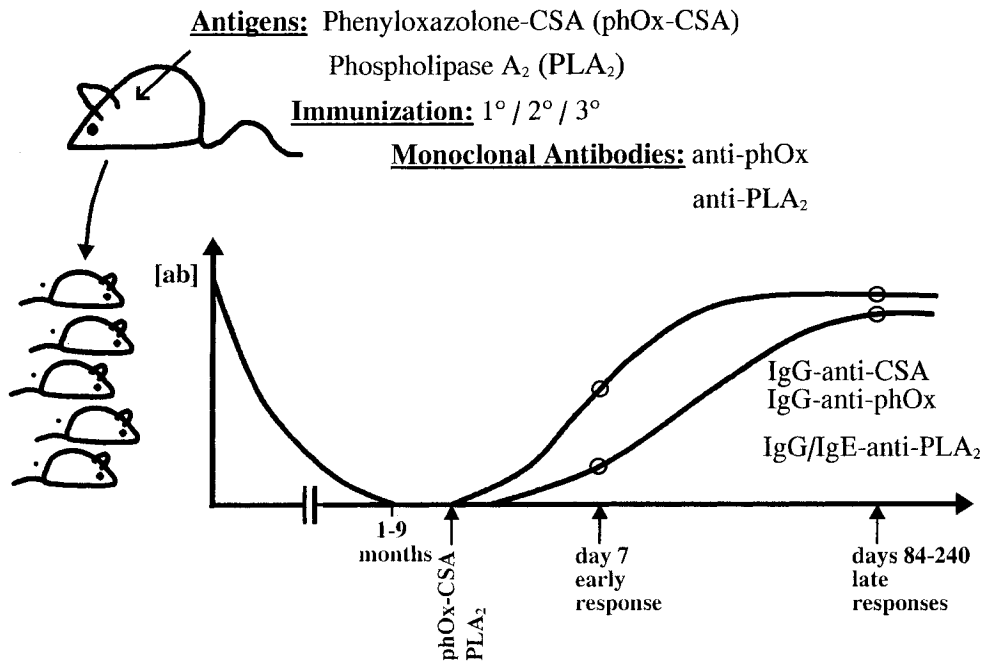

Figure 1: Experimental design (for explanation see text).

Immunisations, production of monoclonal antibodies and analysis of immune responses

Anti-PLA_2 immune response

Immunisation of CBA/J mice was performed as described by *Kolbe* and co-workers (1991) who have shown that minimal doses (MD) of PLA_2 (0.1 μg per injection) leads to a strong IgG-response and small but consistent production of IgE antibodies, while large doses (LD) of PLA_2 induce a strong IgG but no IgE antibody response which can not be reactivated by minimal doses. The determination of IgG and IgE antibody titres in the sera of immunised mice was performed with solid-phase bound sandwich ELISAs as described by *Kolbe* et al. (1991).

Anti-phOx immune response

The immunisations with the thymus-dependent antigen phOx-coupled chicken serum albumin (CSA) have been described recently (*Lemke* et al., 1994; *Lange* et al., 1999). For an analysis of the phOx-reactive primary repertoire, spleen cells of BALB/c mice were fused on day 7 after primary immunisation with the non-secretor X63-Ag8.653 myeloma cells (*Kearney* et al., 1979) by the conventional polyethylene glycol fusion technique. The idiotypic analysis of anti-phOx immune sera and monoclonal antibodies and the sequencing of their variable regions of the H and L chains have been described in (*Lange* and *Lemke*, 1996; *Lange* et al., 1999).

RESULTS

Experimental design

The experimental design is depicted in Figure 1. Female BALB/c mice were immunised 1-3-times (primary = 1°mo,

secondary = 2°mo or tertiary = 3°mo) with the hapten-carrier conjugate phOx-CSA (*Lemke* et al., 1994) or CBA/J females were immunised with the IgE-inducing phospholipase A_2 (PLA_2), the main allergic component of bee venom (*Seeger* et al., 1998). Alternatively, females of both strains were injected with monoclonal anti-phOx or anti-PLA_2 antibodies, respectively. Depending on the maternally-derived anti-phOx, anti-CSA or anti-PLA_2 serum titres, the F1 and F2 generation offspring were allowed to rest until the maternally derived antibodies had vanished from the circulation. Four weeks later, the BALB/c offspring received a primary immunisation with phOx-CSA and the kinetics of the immune response were followed (*Lemke* et al., 1994). Moreover, the quality of the early response on day 7 was analysed with respect to the idiotypic composition of the antisera, i.e. the proportion of Id_{Ox1} antibodies which dominate the early anti-phOx response was determined with Id_{Ox1}-specific monoclonal antibodies. Hybridoma antibodies were also produced on day 7 after primary immunisation and their affinities and the expression of the Id_{Ox1} were analysed and the V(D)J gene sequences of non-Id_{Ox1} antibodies were determined (*Lange* et al., 1999). The offspring of CBA/J dams was immunised with IgE-inducing minimal doses of PLA_2 [primary immunisation with 10 μg and the following injections with 0.1 μg, according to *Kolbe* et al. (1991)] and the IgE and IgG immune responses to PLA_2 were followed (*Seeger* et al., 1998).

Suppression of IgE responsiveness by maternally derived monoclonal IgG antibodies

Twenty years ago, it has already been demonstrated that the IgE immune response to ovalbumin (OVA) in rats can effectively be suppressed by a pre-immunisation of the dams (*Jarrett* and *Hall*, 1979). A variety of experimental conditions indicated that this IgE suppression was, with all likelihood, mediated by IgG-anti-OVA antibodies transferred from the dams to the offspring and that antigen was not involved in the process (*Jarrett* and *Hall*, 1979, 1983, 1986; *Jarrett*, 1984). We have repeated such experiments in mice with another antigen, namely bee venom PLA_2 (*Seeger* et al., 1998), and the results of Jarrett and co-workers could be confirmed and extended: (a) The IgE suppression lasted much longer than IgG-anti-PLA_2 could be detected in the sera of the F1 animals. (b) An IgE response could not be reactivated by repeated immunisations with IgE-inducing minimal doses of PLA_2 for up to half a year. (c) Moreover, we could provide convincing evidence that the IgE suppression by maternally derived IgG works independently of antigen: We prepared anti-PLA_2 monoclonal antibodies which were purified from the culture supernatants by affinity chromatography on Sepharose-immobilised recombinant Protein A or Protein G (rProtein-A- and rProtein-G-Sepharose; Pharmacia, Freiburg i.Br., Germany). These purified IgG-anti-PLA_2 were injected into pregnant CBA/J females 10 days before and 7 days after giving birth to their offspring. It could be demonstrated (Figure 2) that a mixture of ten (nine IgG1 and 1 IgG2b) or even *one* single monoclonal maternally derived IgG-anti-PLA_2 (IgG1 antibody MS613) were equally effective in mediating an IgE-suppression. This suppression of IgE responsiveness was detectable when immunisation with minimal doses of PLA_2 was started at an age of 4 months when low levels of IgE antibodies to PLA_2 were still detectable in the sera (data not shown) as well as when immunisation was started at an age of 8 months when no mater-

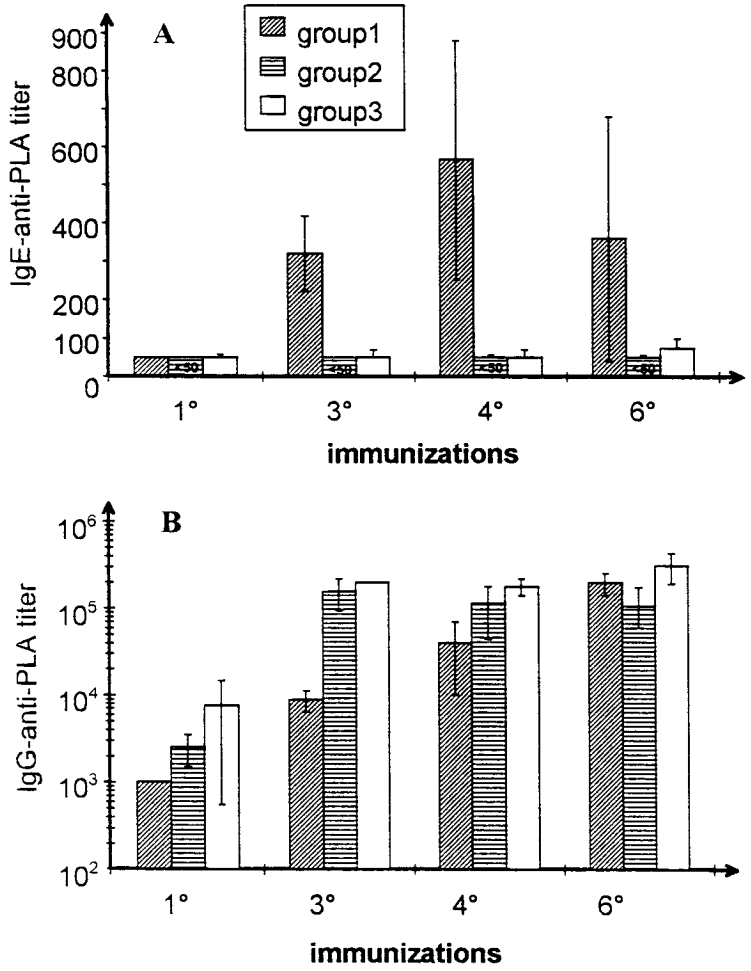

Figure 2: IgE (A) and IgG (B) immune responses of CBA/J mice (n=5-7) born to dams which had received different monoclonal antibodies 10 days before and 7 days after delivery of their offspring. With each injection, the dams received 0.6 mg of one purified antibody or a mixture of antibodies. Dams of group 1 animals had received the monoclonal IgG1-anti-2-phenyloxazolon antibody NQ2/16.2 (*Kaartinen* et al., 1983), dams of group 2 mice had received mAb MS613 as the only IgG1-anti-PLA_2 antibody and dams of group 3 mice had received a mixture of nine IgG1- (including mAb MS.613) and one IgG2b-anti-PLA_2 antibodies. All F1 offspring mice were started to be immunized at an age of *eight* weeks when maternally-derived monoclonal anti-phOx or anti-PLA_2 antibodies could not be detected in their sera. The primary immunization was done with 10 μg PLA_2 while all following immunizations were performed with the IgE-inducing minimal doses of 0.1 μg PLA_2. Two weeks after each immunization, the IgE and IgG titers were determined. The bars indicate the mean ± standard deviation. [Reprinted from *Seeger* et al. (1998) with permission].

nally derived IgG-anti-PLA_2 could be detected, as shown in Figure 2. (d) These experiments demonstrated that one IgG1 antibody was effective to induce IgE suppression. The isotype-dependence of IgE suppression with respect to the other IgG subclasses is currently under detailed investigation with isotype switch variants of the effective monoclonal antibody MS613. Hence,

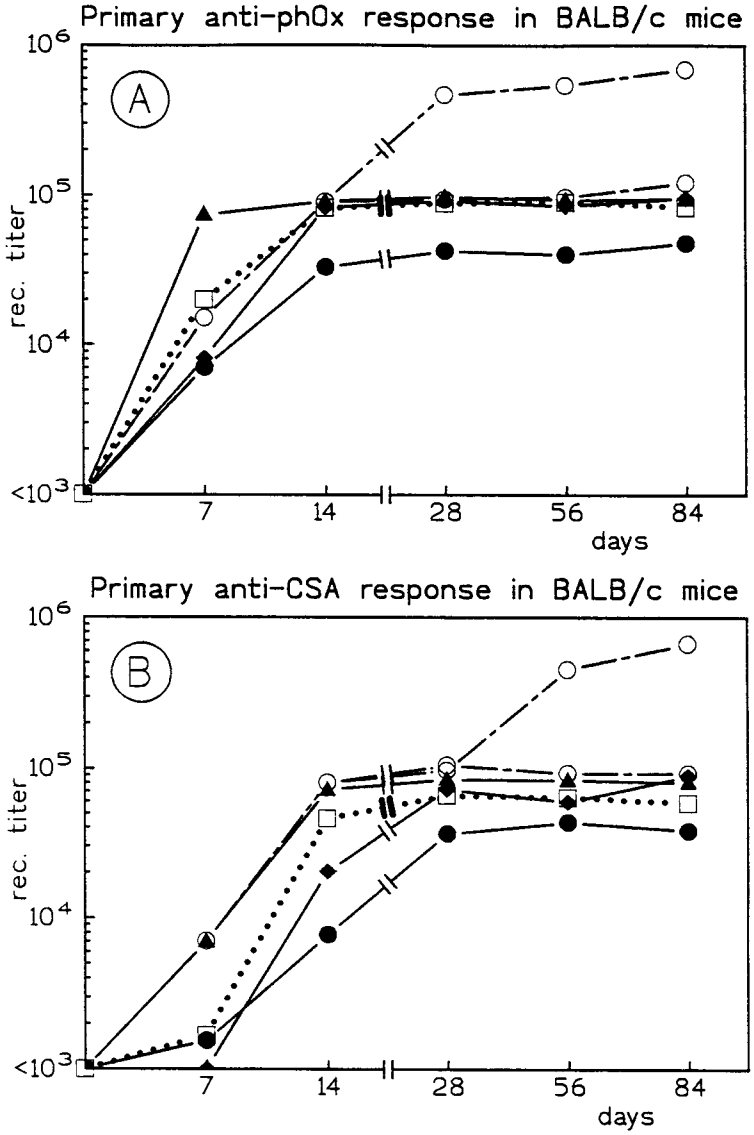

Figure 3: Primary anti-phOx (A) and anti-CSA (B) response in normal BALB/c mice (o·····o), F1-generation offspring born to primarily (s—s), secondarily (u—u) or tertiary (l—l) immunised mothers and F2-generation mice descending from tertiary immunised grandmothers, but non-immunised dams (m—·—m). The primary response was induced with phOx-CSA 4 weeks after complete disappearance of maternal or grandmaternal antibodies from the circulation. Each value indicates the mean titre of a group of mice which consisted of at least 5 and maximally of 17 animals. For the sake of clarity, the standard deviations are omitted here, but included in the comparison for the titres on day 7 and day 84 in Figure 5. For statistical significance see text. The titres were calculated as that serum dilution corresponding to three times cpm background values without antiserum. [Reprinted from *Lemke* et al. (1994), with permission].

our experiments formally prove that antigen is *not* involved in the process and provide an experimental basis to investigate the underlying mechanisms.

Maternal immunisation modulates the primary response to phOx-CSA

For an elucidation of the principle possibility that the immunological experience of the mother may be beneficial for the development of immune responsiveness in the offspring, the kinetics of the primary anti-phOx-CSA immune response were investigated in the offspring of dams which had received a primary (1°mo), secondary (2°mo) or tertiary (3°mo) immunisation. To exclude or at least drastically reduce the possibility that maternally derived antibodies might directly influence this response, the primary immunisation in the offspring was induced 4 weeks after maternal antibodies had declined to background levels of normal mice. The development of anti-phOx and anti-CSA responses in mice born to 1°mo, 2°mo or 3°mo is depicted in Figure 3. In comparison to normal mice, it is striking to see that the early primary response on day 7 of mice born to 1°mo was enhanced ($p<0.001$) whereas it was reduced in mice born to 2°mo ($p<0.001$) or 3°mo ($p<0.001$). Hence, F1 animals born to 1°mo seemed to react with an accelerated response and maximal antibody levels were already reached by day 7 after primary immunisation. In offspring of 2°mo and 3°mo the response was delayed, but mice born to 2°mo reached nearly maximal titres by day 14. In offspring of 3°mo, however, the level of anti-phOx and anti-CSA antibodies was significantly reduced ($p<0.05$) up to 84 days post immunisation.

The development of both antibody specificities was also tested in animals of the F2 generation which received a small amount of antibodies from their grandmothers (*Lemke* et al., 1994). In about half of these F2 mice (45%), the anti-phOx production developed like in normal mice whereas in the other half (55%) of the animals, these grandmaternal antibodies had dramatic effects on the kinetics of the immune response, in that anti-phOx titres continued rising beyond 4 weeks and reached titres of $6x10^5$ at 12 weeks after immunisation (compared to normal mice: $p<0.001$). Such high titres have never been observed before during a primary antibody response to phOx. The anti-CSA response in this subpopulation of F2 mice showed a significant ($p<0.001$) earlier onset as did those animals born to 1°mo. Although the response seemed to reach plateau levels 14 days after immunisation, the anti-CSA titres started to rise again in those 55% of animals and reached, like the anti-phOx response, titres of about $6x10^5$ by day 84.

Maternal tertiary immunisation induces a clonal alteration of the primary day 7 anti-phOx repertoire

Next, it was tested whether the maternal immunisation or the transfer of monoclonal antibodies from the dams to F1 and F2 mice could not only modulate the early day 7 primary anti-phOx antibody response in quantitative terms, but might also induce an alteration of the clonal composition in these early immune sera (*Lange* et al., 1999). For this purpose, the dominant Ox1 idiotype (Id_{Ox1}) was used as a marker. In BALB/c mice, the Id_{Ox1} is quantitatively stable expressed by about 75% of the anti-phOx antibodies during the early primary response (*Kaartinen* et al., 1983a; *Lange* and *Lemke*, 1996; *Lange* et al., 1999). After its early expression, the Id_{Ox1} is lost during the following 2-3 weeks of immune maturation through somatic mutations (*Lange* and *Lemke*, 1996). This is also depicted in Figure 4 (lanes 1 and 2). When BALB/c females were immunised with the IgM-Id_{Ox1} H11.5 two months before mating, IgG-anti-Id_{Ox1} could be detected in the offspring up to an age of 2 months. When these F1 mice were immunised with phOx-CSA after two more months, the

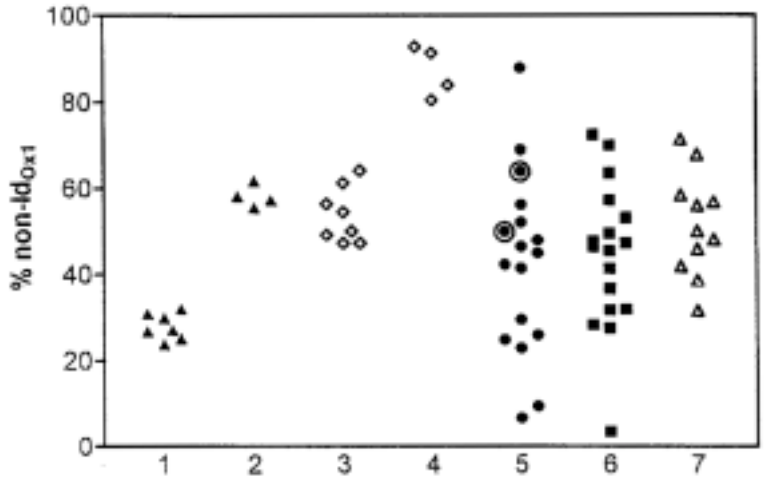

Figure 4: Expression of non-Id_{Ox1} in primary anti-phOx antisera. Normal BALB/c or offspring mice born to mothers or descending from grandmothers immunised in different ways received a primary immunisation with phOx-CSA. The percentage of non-Id_{Ox1} or Id_{Ox1}, respectively, was determined by inhibition with Id_{Ox1}-specific monoclonal antibodies (see section Methods). The percentage of *non*-Id_{Ox1}, is shown for a) primary antisera of normal mice on day 7 (lane 1) and day 14 (lane 2); b) primary day 7 (lane 3) and day 14 (lane 4) antisera of mice born to mothers which had been immunised with the IgM-Id_{Ox1} H11.5; c) primary day 7 antisera of mice born to tertiary phOx-CSA-immunised mothers (lane 5) or descending from tertiary immunised grandmothers, but non-immunised F1 dams (lane 6); d) primary day 7 antisera of mice born to mothers which were injected 2 weeks after mating with a mixture of three highly mutated quaternary anti-phOx antibodies (lane 7). In lane 5 the dots surrounded by circles represent the two mice from which monoclonal day 7 antibodies were produced and whose V(D)J gene sequences were determined. [Reprinted from *Lange* et al. (1999), with permission].

expression of Id_{Ox1}^{+} anti-phOx antibodies in day 7 immune sera was suppressed to about 40% and this proportion decreased to about 10% by day 14 (Figure 4, lanes 3 and 4). Remarkably, as in normal mice (Figure 4, lanes 1 and 2), the expression of the Id_{Ox1} showed little variation between different animals. When the F1 offspring of tertiary immunised dams was analysed in this respect, the pattern of IdOx1 expression was quite different (Figure 4, lane 5). Only 4 out of 17 immune sera showed an Id_{Ox1} expression like those of normal mice (comparison with lane 1). In the majority of 11 mice, the expression of the Id_{Ox1} was suppressed to values of 10-60%. However, 2 mice were observed which even exhibited an enhanced Id_{Ox1} expression to 90-95%. Hence, tertiary immunisation of the dams rendered the Id_{Ox1} expression exceedingly variable.

The proportion of the IdOx1 in day 7 primary antisera was also determined in F2 mice born to non-immunised F1 dams which again descended from females which had undergone a tertiary immune response to phOx-CSA. The content of Id_{Ox1} antibodies in these primary day 7 immune sera (Figure 4, lane 6) varied between individual mice nearly to the same extent as it did in the direct F1 offspring of those tertiary immunised dams (Figure 4, lane 5).

High affinity antibodies derived from late stages of the maturation process represent the highest quality of the mother's immunological experience with regard to a particular immune re-

sponse. It was suspected that they also might influence the outcome of the primary immunisation in the F1 generation. Therefore, the late stages of immune maturation were tried to be imitated by injecting pregnant BALB/c females 2 weeks after mating with a mixture of three anti-phOx monoclonal antibodies (mAb-mo) which were generated from a quaternary immune response. In comparison to the primary Id_{Ox1}^{+} IgG-anti-phOx antibody NQ2/16.2 ($\gamma1,\kappa$), the affinities of these antibodies HL4°/2-16, HL4°/18-22, HL4°/21-3 (all $\gamma1,\kappa$) were enhanced by factors of 1100, 120 and 550, respectively, and their V/D/J gene expression have been described (*Lange* et al., 1999). Four weeks after the disappearance of these maternally derived antibodies from the circulation, the F1 animals received a primary immunisation with phOx-CSA and again, the proportion of the Id_{Ox1} among the anti-phOx humoral antibodies in day 7 antisera was determined. Strikingly, the expression of the Id_{Ox1} varied between individual mice (Figure 4, lane 7) nearly to the same extent as in the two former groups, i.e. 3°mo/1°Fl animals (lane 5) and 3°mo/1°F2 mice (lane 6). Moreover, in comparison to normal mice (lanes 1 and 2) and to the offspring born to IgM-Id_{Ox1}-immunised dams (lane 3 and 4), it was evident that the proportion of Id_{Ox1} varied to a much greater extent in the F1 and F2 offspring of tertiary immunised dams as well as in F1 mice which received the mixture of high-affinity quaternary anti-phOx antibodies.

The pre-immune anti-phOx repertoire of mice born to tertiary immunised dams contains antibodies with enhanced affinities

For an analysis of the quality of the primary antibody repertoire of mice born to tertiary immunised dams, the F1 offspring were allowed to rest until their maternal anti-phOx humoral antibody titres had vanished. Eight weeks later, the mice received a primary immunisation with phOx-CSA. Hybrid cell lines secreting anti-phOx antibodies were produced on day 7, a time point at which in normal mice practically no somatic mutations have been observed (*Berek*, 1992). From two of these 3°mo/1°F1 mice, born to two dams, 55 (23+32) anti-phOx antibody-secreting hybridomas were produced. Forty-two (14+28) of them secreted sufficient amounts of antibody for further analysis. Typing with specific monoclonal anti-IdOx1 antibodies (*Lange* et al., 1999) revealed that 23 (9+14) of them, corresponding to about 55% (64% and 50%), were non-Id_{Ox1} (Table 1). This proportion corresponded to the content of non-Id_{Ox1} observed in the sera of these mice (Figure 4, lane 5, circled dots). The relative affinities of these antibodies were measured with a hapten binding inhibition test (Table 1) and compared with that of the prototype IgG-Id_{Ox1} NQ2/16.2 (Kaartinen et al., 1983b). Two randomly chosen Id_{Ox1}^{+} antibodies from one of the two mice (group 1 antibodies JL2/24-22 and JL2/26-14) had nearly identical affinities as mAb NQ2/16.2 (Table 1). Twelve (52%) of the 23 non-Id_{Ox1} antibodies from our 3°mo1°F1 mice exhibited enhanced affinities in comparison to non-Id_{Ox1} antibodies in normal mice (*Pelkonen* et al., 1986). Compared to the IgG-Id_{Ox1} NQ2/16.2 (Table 1), the relative affinities of group 2 antibodies (n=5, ~22%, JL1/1-1, JL1/1-10, JL1/13-8, JL2/18-7 and JL2/19-3) were even 7-25-times higher, and those of group 3 antibodies (n=7, ~30%; JL1/1-17, JL1/3-10, JL1/11-7, JL2/19-6, JL2/27-13, JL2/28-18, JL2/29-3) were in the same range as the IgG-Id_{Ox1} NQ2/16.2 (factors of 0.9-1.9). Another group of non-Id_{Ox1} antibodies (n=11, ~48%) exhibited considerably lower affinities than the IgG-Id_{Ox1} NQ2/16.2. Group 4 antibodies JL1/14-22, JL2/18-

1 and JL2/25-5 (Table 1) belong to this group and exhibited 4-10-times lower affinities (factors of 0.152 to 0.28) than mAb NQ2/16.2. In normal mice, the Id_{Ox1}-negative antibodies of the primary anti-phOx response are of 10-100-times lower affinity than the dominant Id_{Ox1} antibodies (*Pelkonen* et al., 1986). Hence, the enhanced affinities of primary non-Id_{Ox1} antibodies in the offspring of tertiary immunised dams seem to indicate that this maternal pre-immunisation is able to select a hitherto unknown B cell population to the pre-immune repertoire which can, in contrast to normal mice, be activated by the thymus-dependent hapten-carrier complex phOx-CSA.

Variable region sequences of non-Id_{Ox1} antibodies in the offspring of tertiary immunised dams

Since such high affinities of primary day 7 anti-phOx antibodies have so far not been observed for Id_{Ox1}-negative antibodies, the V region sequences of these antibodies were determined. They are depicted in Table 1 and have been described in Lange et al. (1999). The key features of the expressed genes can be summarised as follows:

(1) *None of the non-Id_{Ox1} antibodies derived from Id_{Ox1} antibodies by somatic mutations.*

Although some antibodies of groups 2 and 3 in Table 1 are encoded by the Id_{Ox1} genes V_κOxl (JL1/13-8, JL2/18-7, JL2/19-3) and/or the V_HOxl gene (JL2/19-6, JL2/18-7, JL2/27-13, JL2/28-18) they were idiotypically Id_{Ox1}-negative and the coding variable genes contained no somatic mutations which were identical to known affinity-increasing substitutions. We take this as an indication that maternally derived antigen phOx-CSA is not the driving force for the early activation of these antibodies. This view is corroborated by the fact that these antibodies were established from a suppressed primary response (see Figure 2) which showed in no way typical secondary kinetics.

(2) *Non-Id_{Ox1} of the early primary response in maternally-manipulated F1 mice may be encoded by V_H and V_L genes and combinations thereof which are typical of memory responses in normal mice.*

Some of the non-Id_{Ox1} from the early primary response of 3°mo/F1 mice were encoded by variable region genes which previously have been found in memory responses of normal mice:

a. The V_H of JL2/19-3 has previously been observed in the secondary antibody NQ10/12.5 (*Berek* et al., 1985).
b. The V_H of JL1/13-8 was also expressed in the tertiary anti-phOx antibody NQ22/56.1 (*Berek* and *Milstein*, 1987; *Berek* et al., 1987).
c. and d. Both antibodies JL1/11-7 and JL2/29-3 were encoded by V_H6 U21563 which has already been observed in the secondary antibody NQ10/2.12.4 (*Berek* et al., 1985).
e. Group 3 antibody JL2/28-18 is encoded by unmutated V_κ8 and V_HOxl genes. The V_κ8 gene was also expressed by one of our quaternary anti-phOx antibody, however, in connection with a highly mutated V_HOxl (Table 1). Hence, the unmutated V_HOx1/V_κ8 germline configuration is the fifth example of an early appearance of a gene combination which in normal mice was first observed during a memory response.

(3) *Non-Id_{Ox1} may be encoded by new germline genes.*

The V_κ1 gene coding for antibody JL2/19-6 probably represents a new germline gene.

(4) *Early primary non-I_{Ox1} may be encoded by known germline genes hitherto unknown in the anti-phOx response of normal BALB/c mice.*

Some of the primary day 7 antibodies in the offspring of tertiary immunised

dams expressed V_L genes and V_H/V_L gene combinations which have so far not been described for any phase of the immune maturation in normal mice. The V_κ45.1 of the V_κ1 family has so far only been found in an essentially unmutated form in normal mice during the anti-phOx response. In contrast, three non-Id_{Ox1} of group 3 are encoded by V_L germline genes of the V_κ1 family which are new for the anti-phOx response, i.e. JL1/1-17 and JL2/27-13 by the V_κ1 gene M28131 and, as mentioned above, the V_κ1 gene of antibodies JL2/ 19-6 probably represents a new germline gene. Moreover, the V_κ2 gene coding for the high affinity antibody JL1/1-10 has also not been observed during any phase of the immune maturation in normal mice. In contrast, group 4 non-Id_{Ox1} antibodies exhibited affinities in the range of non-Id_{Ox1} antibodies from normal mice (*Pelkonen* et al., 1986). Three members of this group have been sequenced (JL1/14-22, JL2/18-1 and JL2/25-5 in Table 1) and were found to be encoded by the V_L genes $V_\kappa$ars, V_κ45.1 and V_κ8 respectively which have also been found in normal BALB/c mice 7 days after primary immunisation (*Kaartinen* et al., 1986).

(5) *Non-Id_{Ox1} may be encoded new V_H/V_L combinations from different gene families*.

Moreover, in the offspring of tertiary immunised dams (3°mo/1°F1), some of the early primary non-Id_{Ox1} with higher (group 2) and equal (group 3) affinities as Id_{Ox1} antibodies were encoded by new V_H/V_L gene combinations. In normal mice, the V_HOxl (V_H2 gene family) is associated either with V_κOxl (V_L4/5 family) or $V_\kappa$ars (V_L10 family) (*Berek* et al., 1987), whereas in 3°mo/1°F1 mice two new combinations were found: In antibody JL2/27-13 the V_HOxl gene is associated with a member of the V_κ1 family (M28131) and in antibody JL2/28-18 with a member of the V_κ8 family (M34616) (Table 1). Id_{Ox1}-negative antibodies in normal BALB/c which express the V_HOxl/$V_\kappa$ars gene combination have lower affinities than the Id_{Ox1} NQ2/16.2 (V_HOx1 / V_κOxl). In contrast, the two antibodies from 3°mo/1°F1 mice have either an equal (JL2/27-13 with V_HOxl/V_κ1) or even a slightly higher affinity (JL2/28-18 with V_HOxl/V_κ8) than the IgG-Id_{Ox1} antibody NQ2/16.2 (Table 1). The Id_{Ox1}-negative antibodies JL2/25-5 (Table 1, group 4) and JL1/1-10 (Table 1, group 2) provide even more impressive examples for an affinity enhancement by new gene combinations. Antibody JL2/25-5 is encoded by a V_H1 gene in association with a V_κ8 gene which is also known from the anti-phOx response in normal mice, while the V_H1 of mAb JL1/1-10 is associated with a V_κ2 gene. This gene combination confers a 56-times higher affinity than the "normal" combination V_H1/V_κ8 in JL2/25-5. Hence, among other reasons, the higher affinities of a considerable proportion of non-Id_{Ox1} antibodies in the offspring of tertiary immunised dams may also be due to a selection of new V_H/V_L gene combinations.

Does maternally mediated immunomodulation activate the natural IgM pool?

In the offspring of secondarily immunised BALB/c dams (2°mo), maternal IgG antibodies to the hapten phOx and the carrier CSA could be detected up to an age of about 4 months (*Lemke* et al., 1994). In the sera of these F1 mice (n=20), phOx-specific antibodies were further tested for up to 8 months and it became evident that anti-phOx titres increased again. At an age of 8 months, all animals exhibited titres which were higher than in a control group of normal mice born to non-im-

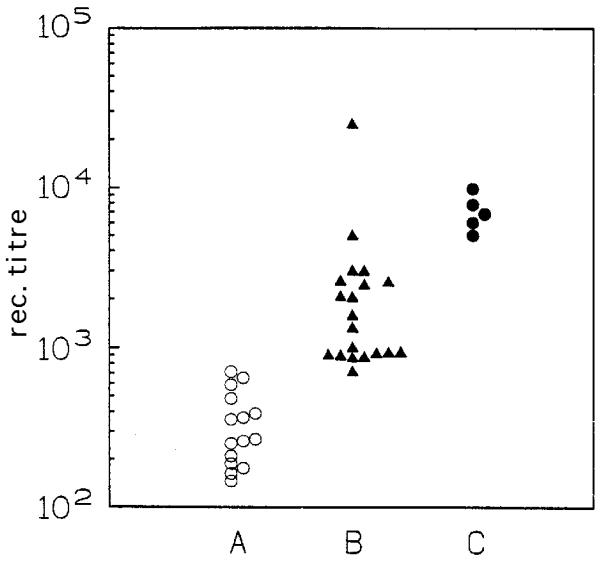

Figure 5: Comparison of anti-phOx IgM serum titers in (A) normal non-immunized BALB/c mice (8 months old, n = 15), (B) non-immunized F1-generation offspring (8 months old, n = 20) born to secondarily immunized dams and (C) normal BALB/c mice (n = 5) on day 78 after primary immunization with phOx-CSA. [Reprinted from *Lemke* et al. (1994), with permission].

munised dams (Figure 5A, n=15, $p<0.001$) and these anti-phOx antibodies turned out to be of the IgM class (Figure 5B) . As a control, the level of IgM anti-phOx serum titres in a group of normal BALB/c mice (n=5) 7 days after primary immunisation with phOx-CSA is also shown (Figure 5C). Since the production of IgM anti-phOx in the offspring of secondarily immunised dams was not followed by switch to IgG antibodies, it may be asked whether the enhanced level of IgM indicate an activation of natural antibodies.

DISCUSSION

Isotype regulation by maternal IgG antibodies

Earlier experiments in the rat had already provided solid evidence that maternally derived IgG antibodies have the capacity to suppress an IgE response in the offspring (*Jarrett* and *Hall*, 1983) and our own data confirm this conclusion (see above and *Lemke* et al., 1994). Moreover, it could be demonstrated that maternally-derived exogenous monoclonal IgG antibodies, i.e. without participation of antigen, suppressed the IgE response to PLA_2 in CBA mice to the same extent as does an active immunisation of the dams. Strikingly, the transfer of a *one* single IgG-anti-PLA_2 was as effective as a mixture of ten of these antibodies or an active immunisation of the mothers. It is highly unlikely that one IgG mAb can cover the whole range of antigenic determinants on PLA_2. Moreover, it has been shown that IgE- and IgG-anti-PLA_2 antibodies recognise non-cross-reactive determinants when induced by immunisation with MD doses of PLA_2 (*Kolbe* et al., 1995). Therefore, it can be concluded that maternally derived IgG antibodies do not act by simply masking antigenic determinants of PLA_2. This is supported by experiments

which demonstrated that mAb MS.613 did *not* inhibit the binding of a monoclonal IgE-anti-PLA_2 to its plastic-bound antigen (data not shown). Moreover, it was tested whether PLA_2 which was injected at high concentration (100 μg) into pregnant CBA/J female mice together with a IgG-anti-PLA_2 mAb (300 μg) could be transferred to the offspring. However, neither free PLA_2 nor a primary anti-PLA_2 immune response could be detected in F1-generation mice, thus allowing the conclusion that neither free nor antibody-bound PLA_2 antigen was transferred from the dams to the offspring. These results are in line (a) with those of *Jarrett* and *Hall* (1979) who observed that treatment of the dams with ovalbumin alone (in the absence of adjuvant could in no way influence a subsequent IgE response of the offspring, and (b) with the finding that also immune complexes are not transferred to the foetus via the placenta (*Wood*, 1994). The data obtained in rats (*Jarrett* and *Hall*, 1986) and in the mouse (*Seeger* et al., 1998) demand for an investigation of possibility to suppress IgE responsiveness by maternally derived IgG antibodies in humans (*Jarrett*, 1984).

Since it has been assumed that decisive events for the development of allergy or asthma in man occur early in life (*Holt*, 1994, 1995; *de Weck* et al., 1995), the conclusion may be valid that a quantitatively or qualitatively insufficient transfer of maternal IgG antibodies to the babies may be at least one possible cause. This in turn would favour the idea that mothers should experience as many environmental allergens as possible and at concentrations which stimulate strong IgG responses. This view is in line with the conception that deficits in immunological stimulations, in particular by T_H1-stimulating microbial infections, is one possible cause for the increase in atopic allergies in highly developed countries (*Rook* and *Stanford*, 1998) and may as well have been decisive for the differential development in allergic and asthmatic diseases in East and West Germany (*Wichmann*, 1996).

Maternally modulated kinetics of primary immune responses

Our investigations started from the idea that maternal high-quality immunological experience would induce beneficial effects in the immune system of the new-borns. However, it was not clear as to how such effects could be detected. Therefore, we chose the well-studied anti-hapten immune response of BALB/c mice to the hapten-carrier conjugate phOx-CSA and expected to see alterations of any of the characteristic features of this response which have been summarised in *Lange* et al. (1999). The analysis of the primary response to phOx-CSA at an age when no maternal antibodies could be detected above normal background levels seemed to support our hypothesis: (a) In the F1 offspring of primarily immunised dams, the primary response to phOx as well as to CSA developed faster and this also held true for the anti-CSA response in F2-generation mice (Figures 3A and B). (b) In the offspring of 2°mo, the primary responses to phOx and CSA were delayed, but reached maximal levels of normal mice. In 3°mo/1°F1 mice, however, both responses were not only delayed, but remained at lower levels. (c) A striking results was the observation that about half of the F2-generation mice born to non-immunised F1 mice developed a continuing or a second increase during the late phases of the primary anti-phOx as well as anti-CSA responses. Especially the latter two observations that the maternal immunisation even showed early and late immuno-modulatory effects in the F2 generation argue against the possibility that antigen transferred from the grandmothers via the F1 females to the F2 offspring might be responsible.

A particularly interesting point is the difference in primary responses of F1 mice born to 3°mo and their F2-generation offspring. We are inclined to regard the suppressed response in the F1 animals to indicate a *supra*optimal stimulation, whereas the F2 mice may have received a nearly optimal stimulation after an appropriate dilution of the relevant antibodies in the F1 mice. This may be related to the observation that anti-idiotypic antibodies may enhance the corresponding idiotype at low doses, but suppress it at higher doses (*Kelsoe* et al., 1981).

Maternally induced alteration of the primary repertoire

The idiotypic analysis of the early day 7 primary immune response in F1- and F2-generation mice indicated that high-affinity antibodies of tertiary or quaternary anti-phOx responses are able to alter the clonal composition of the available phOx-reactive repertoire (Figure 4) (*Lange* et al., 1999). Since the tertiary immune and the quaternary monoclonal anti-phOx antibodies which were transferred from the dams to the offspring, exhibited an extremely weak cross-reactivity with the dominant Id_{Ox1} of the primary response (*Lange* et al., 1999), the question arose as to how antibodies from the late stages of immune maturation could interfere with the establishment of the primary repertoire. The genetic analysis revealed that the maternal influence caused a strong affinity enhancement in the non-Ox1 idiotypic antibody repertoire (Table 1) and it seems unquestionable that this affinity increase is *not* induced by antigen which might have been transferred from the dams to the offspring. This view is based on the fact that no known affinity increasing somatic mutations could be observed in the primary anti-phOx antibodies of 3°mo/1°F1 mice. This finding seems to contrast with the idea that idiotype suppression might be important for the selection of "*somatic antibody mutants . . . through suppression of the wild type*" which might operate already "*in the pre-immune repertoire, generating mutants before contact with antigen*" (*Cumano* and *Rajewsky*, 1986). If this mechanism is not responsible for the altered expression of the major Id_{Ox1} in the offspring of maternally manipulated dams, it has to be asked how else this outcome may be induced by the extremely weak cross-reactivity of high-affinity quaternary anti-phOx antibodies with the Id_{Ox1}. Two possible mechanism may be envisaged. The first rests on investigations performed by Klinman and co-workers (*Linton* et al., 1989; *Klinman*, 1996) who observed that naive and memory B cell progenitors participating either in the primary or memory responses respectively, can have different requirements for activation. Hence, it is conceivable that maternal antibodies might induce a pre-activation of the B cell clones in the memory compartment so enabling them to participate already in the primary response. This possibility may be indicated by our finding that some of our non-Id_{Ox1} antibodies from 3°mo/1°F1 mice were encoded by V(D)J gene combinations which in normal mice have been found during secondary to quaternary memory responses. Secondly, it has been demonstrated that the specificity of the membrane-bound immunoglobulin may be altered by receptor editing via successive recombinations (*Levy* et al., 1989; *Radic* and *Zouali*, 1996). It is conceivable that maternal antibodies might influence the establishment of the B cell repertoire by interference with the association of productive V_L and V_H gene recombinations. In the present context it is especially intriguing that an editing of the B cell antigen receptor can be induced by anti-idiotypic antibodies (*Levy* et al., 1989, 1998; *Hertz* and *Nemazee*, 1997) and that this process may be induced by

Table 1: Idiotype, affinity and VL- and VH-gene expression of anti-phenyloxazolone antibodies from maternally manipulated mice.

Clone[a]	Group[b]	Id_{Ox1}[c]	relative affinity[d]	affinity factor[e]	V_L-gene[f]	J_κ[g]	V_v-gene[f]	J_H[g]
NQ2/16.2	1	+	7.0×10^{-3}	1	$V_\kappa4$ (V_κOx1) Z22128*[h]	5*	V_H2 (V_HOx1) K00724*	3*
JL2/24-22	1	+	8.6×10^{-3}	0,81	$V_\kappa4$ (V_κOx1) Z22128*	5*	V_H2 (V_HOx1) K00724*	3*
JL2/26-14	1	+	8.3×10^{-3}	0,84	$V_\kappa4$ (V_κOx1) Z22128*	5*	V_H2 (V_HOx1) K00724*	3*
JL1/1-1	2	–	1.0×10^{-3}	7	n.d.	n.d.	n.d.	n.d.
JL1/1-10	2	–	4.8×10^{-4}	14.6	$V_\kappa2$ M34622*	5*	V_H1 S73918*	2*
JL1/13-8	2	–	2.8×10^{-4}	25	$V_\kappa4$ (V_κOx1) Z22128	5*	V_H1 M36225	4*
JL2/18-7	2	–	4.8×10^{-4}	14.6	$V_\kappa4$ (V_κOx1) Z22128*	5*	V_H2 (V_HOx1) K00724*	4*
JL2/19-3	2	–	3.8×10^{-4}	18	$V_\kappa4$ (V_κOx1) Z22128*	5*	V_H5 U04229*	3
JL1/1-17	3	–	7.7×10^{-3}	0.9	$V_\kappa1$ M28131*	1*	V_H1 M15224*	4
JL1/3-10	3	–	5.3×10^{-3}	1.3	$V_\kappa1$ (V_κ45.1) X66640	2	V_H1 Z73349*	4*
JL1/11-7	3	–	7.4×10^{-3}	0.9	$V_\kappa1$ (V_κ45.1) X66640*	5*	V_H6 U21563*	3*
JL2/19-6	3	–	6.6×10^{-3}	1.06	$V_\kappa1$ M28131, new germline gene[i]	2	V_H2 (V_HOx1) K00724*	3*
JL2/27-13	3	–	6.6×10^{-3}	1.06	$V_\kappa1$ M28131*	1*	V_H2 (V_HOx1) K00724*	3*
JL2/28-18	3	–	3.6×10^{-3}	1.94	$V_\kappa8$ M34616*	5*	V_H2 (V_HOx1) K00724*	4*
JL2/29-3	3	–	4.5×10^{-3}	1.55	$V_\kappa1$ (V_κ45.1) X66640	1*	V_H6 U21563*	3*
JL1/14-22	4	–	4.6×10^{-2}	0.152	$V_\kappa10$ M54905*	1	V_H1 M15224*	4
JL2/18-1	4	–	4.3×10^{-2}	0.163	$V_\kappa1$ (V_κ45.1) X66640	2*	V_H1 S73918*	3
JL2/25-5	4	–	2.5×10^{-2}	0.28	$V_\kappa8$ M34616*	5*	V_H1 S73918*	3

ultralow affinity ligands (*Lang* et al., 1996). Hence, it seems possible that our high affinity tertiary or quaternary antibodies which are extremely weakly cross-reactive with Id_{Ox1} antibodies might activate network components which in turn interfere with the normal regulation of the major Id_{Ox1}. Moreover, an activation of the receptor editing can be recognised by L-chain rearrangements (*Hertz* and *Nemazee*, 1997), an observation which may well be related to our finding that the L-chain repertoire of the anti-phOx response is considerably increased in F1 mice born to immunised dams (see above). Thirdly, another striking observation was the induction of IgM anti-phOx antibodies in non-immunised offspring of secondarily immunised dams. Since the production of these IgM antibodies was not followed by a switch to IgG, it is tempting to speculate that those IgM belong to the pool of natural antibodies. This possibility is under current investigation by analysing the phOx-reactive repertoire of $CD5^+$ B lymphocytes. The induction of IgM antibodies by maternal influence has been confirmed in the anti-arsonate response by showing that maternal Ab1 induced IgM anti-arsonate antibodies in the offspring without immunisation of the F1 animals (*Ismaili* et al., 1995).

Finally, it may be questioned whether the observed beneficial effects of a maternal immunisation for the development of immune responsiveness in the offspring may also be of relevance for far back dating immunological experiences of the mother and not only for immune responses which happen shortly before or during pregnancy. In this respect is important to consider recent investigations which have demonstrated that memory of the B cell compartment does not only depend on memory cells, but also on long-lived plasma cells in the bone marrow (*Manz* et al., 1997). Importantly, the survival of these long lived plasma cells is independent of antigen (*Manz* et al., 1998). Hence, it

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a: All antibodies from the JL1- and JL2-fusions were of the γ1/κ isotype.

b: The antibodies have been arranged into groups according to their Id_{Ox1} expression and affinity compared to the reference antibody NQ2/16.2 (6, 39, 41).
Group 1: Id_{Ox1}^+ with affinities as the NQ2/16.2; group 2: *non*-Id_{Ox1} with higher affinities; group 3: *non*-Id_{Ox1} with affinities as NQ2/16.2; group 4: *non*-Id_{Ox1} with lower affinities.

c: The idiotyping of the antibodies has been performed with Id_{Ox1}-specific monoclonal anti-bodies 8-21/W18 and 6-8/R20 as described (64); see also legend to Figure 1.

d: The indicated values represent the dilutions of a 0,0035 M stock solution of phOx-cap which inhibited the binding of pre-determined identical amounts of anti-phOx antibodies to solid phase-bound phOx bovine serum albumin by 50%.

e: The affinity factor indicates the quotient of the relative affinity of NQ2/16.2 divided by that of a particular antibody, i.e. antibodies with an affinity factor of >l exhibited a higher affinity while those with an affinity factor of <l have a lower affinity than NQ2/16.2.

f: The analysis of the light and heavy chain variable genes to gene families has been performed with computer programs (see section Materials and Methods) and according to *Dildrop* (1986). When possible, the trivial names of genes are indicated in brackets.

g: The J gene segments of the L- and H-chain are indicated by numbers.

h: The asterisk (*) indicates an identical known germline gene indicated by its reference number of the European Bioinformatic Institute (EBI). An EBI number without an asterisk indicates the next related, but *not* identical germline gene.

i: The appraisal as a new germline gene relies on the sequence comparisons, but is not proven at the genomic level.

n.d.: not determined

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can be assumed that a large proportion of serum IgG antibodies is the product of these bone marrow plasma cells and can be transferred to the offspring for a long period of time. This favours the idea that maternal antibodies really represent a large proportion of the entire immunological experience of the mother and have the potential to guide the education of the neonatal immune system.

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IMMUNOLOGICAL SPECIFICITY, INTERNAL IMAGES, AND THE ORIGINAL IDIOTYPIC SIN

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SUMMARY

The idiotype network hypothesis of Jerne led to the observation that some anti-idiotypic antibodies could mimic antigens reactive with the idiotype-expressing antibody. While such findings prompted some investigators to classify anti-idiotypic antibodies into relatively rigid classes, members of which either did or did not mimic antigen, consideration of relevant physical chemical principles suggests that mimicry should be more similar to a quantitative than a discrete variable. In addition, it is reasonable to expect that there are many ways to assess the extent of mimicry and these various measures of mimicry might not correlate perfectly. Immunochemical studies of antibodies specific for the cell wall polysaccharide of group A streptococcus and anti-idiotypes for these antibodies, as well as structural studies of diverse complexes between idiotypic and anti-idiotypic Fab or Fv fragments, are consistent with these conclusions.

INTRODUCTION

It is now more than four decades since *Fazekas de St. Groth* and *Webster* (1966a, b) reported their findings pertaining to the immunological phenomenon referred to as Original Antigenic Sin, the discovery of which they credited to *Francis* (1953). The basis of Original Antigenic Sin was the finding that vaccinees, previously infected by type A influenza virus, produced antibodies specific for the immunising influenza vaccine antigens but these antibodies reacted better with (or were of higher titre for) the antigens corresponding to the virus associated with the primary exposure through infection. Thus, Original Antigenic Sin refers to an immunological situation where the patterns of subsequent responses were significantly shaped by an initial immunological experience. In what follows, I suggest that the prior exposure of immunologists to certain concepts related to the phenomenon of idiotypy shaped much subsequent thinking about the nature of idiotypic recognition and mimicry of antigen by anti-idiotypes. It is appropriate to associate the word "sin" with these early notions because they were based on simplistic views of non-covalent interaction that have tended to hinder the adoption of more realistic conceptions of antigen recognition and anti-idiotypic mimicry.

THE IDIOTYPE NETWORK

In 1974, Niels Jerne published an extraordinarily influential review, in which he developed a then-novel view of the immune system as a network of clones functionally linked to each other through receptor-receptor (idiotype-anti-idiotype) interactions. Jerne's vision was exhilarating, transforming the immune system from a mere mechanism based on clone-versus-clone competition into a highly integrated, co-operative system with echoes of the central nervous system. Over the next two decades, many of the experimental directions stimulated by the network hypothesis proved equivocal if not futile. Ultimately, the immunological community grew tired of the unlimited layers of anti-idiotypic recognition that seemed pliable enough to rationalise any result, once obtained, but of limited value for prediction (*Greenspan*, 1997a). Nevertheless, there are concepts (e.g., molecular mimicry) and experimental results (e.g., identification of clonal populations of lymphocytes *in vivo*) pertaining to idiotype-anti-idiotype interactions that continue to be accepted and that relate to the rest of immunology and biology.

Central to Jerne's conception of the idiotypic network was the idea that in addition to antigen, any antibody variable (V) module (heavy and light chain variable domains), could bind or be bound by one or more other antibodies, termed anti-idiotypic antibodies (anti-Ids or Ab2). One can imagine that the pattern of thought that led Jerne to this notion bears a resemblance to the thinking that led others to the concepts of synthetic (*Geysen*, 1984; *Houghten*, 1985) or phage display peptide libraries (*Scott* and *Smith*, 1992). The central insight is that molecular recognition is a quantitative matter, and that it cannot reach perfection (*Naray-Szabo*, 1993). For example, intrinsic (monovalent) affinities of antibodies for antigens can range at least from 10^5 to 10^{11} L/M (*Karush*, 1978).

The followers of Jerne went on to classify anti-idiotypic antibodies into several categories based on the locations of the sites that they recognised on the idiotypic V module (reviewed in *Gaulton* and *Greene*, 1986). An anti-Id that bound to a site (idiotope) not overlapping with the antigen-binding site (or paratope) was to be called an α anti-Id or Ab2α. An anti-Id that bound to a site that overlapped with the antigen-binding site was to be called a β anti-Id or Ab2 It was assumed that the β-type anti-Ids would be able to mimic the antigen with respect to the idiotypic antibody. It should be noted that additional categories of anti-Ids have been proposed (*Gaulton* and *Greene*, 1986), but these categories did not find extensive use in the experimental literature.

INTERNAL IMAGES

The above ideas were appealing and easily grasped, and they stimulated an enormous wave of research designed to address how the idiotype network operated and how different kinds of anti-Ids contributed to this system. Of special interest were the β anti-Ids or Ab2β, also referred to as *internal images* by Jerne, which were believed to be important both for understanding the physiology of the idiotypic network and for clinical applications of anti-idiotypic antibodies, such as vaccine development (*Nisonoff* and *Lamoyi*, 1981; *Roitt* et al., 1981). It is perhaps paradoxical that the insights which led Jerne to explore

the implications of the previously described phenomenon of idiotypy were apparently abandoned in some of his conceptions relating to internal images and the mimicry of antigen by anti-Ids. Specifically, the conception of the internal image was developed as if guided almost solely by notions of molecular shape. The unfortunate consequences of this shape-centred thinking are chronicled in hundreds of articles in the immunology literature that appeared between the mid 1970s and the late 1980s.

What was largely missing in Jerne's conception of the internal image was the framework provided by the principles of physical chemistry, especially thermodynamics. This gap in Jerne's thinking may not seem surprising given that other immunologists were (are) in the habit of failing to incorporate such physical principles in their thinking. What makes it surprising, is that Jerne had done experimental work, early in his career, on the strength of antibody-antigen interaction, and had a long-term interest in physical chemistry (*Tauber*, 1994). It may well be that Jerne himself understood the limitations of his concept of the internal image, but many of those who were stimulated by his ideas appear to have been less aware of the caveats.

THE PHYSICAL BASIS OF BIOMOLECULAR RECOGNITION

The driving force for the formation of a non-covalent complex between biological macromolecules, or between a macromolecule and a small molecule, is the free energy change associated with the formation of the complex. What is often insufficiently appreciated is that this free energy change is derived from and applies to the entire chemical system in which the ligand or antigen (e.g., Id) and the receptor or antibody (e.g., anti-Id) are components. The solvent, counterions, and other solutes all can influence the free energy change for a particular interaction. Thus, the tendency of investigators to rationalise the intrinsic affinity between two molecules solely by analysis of the structural details of the intermolecular interface is to be discouraged despite its usefulness in some circumstances (*Greenspan*, 1992a). In the general case, conclusions relating to intermolecular affinity and derived only from information pertaining to the structural features of the complex will not be reliable.

Appreciation of another feature of the free energy change of complex formation is crucial to clear thinking about molecular recognition. The free energy change of complex formation reflects the relative stabilities of reactants and products. This point carries profound implications for any effort intended to determine the individual contributions of amino acids, atoms, or other sub-units of structure to a particular non-covalent interaction. The key point is that a mutation that has no direct effect on the energetics of the complex between antibody (receptor) and antigen (ligand), but that destabilises a reactant in the unbound state, will have the effect of increasing the affinity of the interaction. For some purposes, it may not be necessary to distinguish such mutations from those that directly stabilise the complex, while in other cases the distinction may well be important.

What, then, is an epitope, or antigenic determinant? The standard answer is that an epitope consists of the identities and spatial co-ordinates of the molecular sub-units (e.g., amino acids or atoms) that make physical contact with an antibody or T cell receptor. The problem with this definition is that in many cases, the immunologist is primarily interested in knowing which molecular sub-units contribute energeti-

cally to the cognate interaction or to the discrimination between cognate and non-cognate ligands (*Van Regenmortel*, 1989). These sets of sub-units are not necessarily identical with each other or with the set of contact residues (*Greenspan*, 1992a, 1997b). Thus, if the real interest is in these sets of energetic contributors, or in the residues for which substitution (through mutation) affects the affinity of binding or the differential affinities of binding for two or more ligands, then defining the contact residues is, in the general case, a poor substitute.

MIMICRY AS A QUANTITATIVE, MULTIDIMENSIONAL VARIABLE

How do these considerations pertaining to the nature of non-covalent interactions and epitopes affect an understanding of anti-idiotypic mimicry of antigen, or of molecular mimicry in general? An appropriate starting point is to consider what mimicry of an antigen (epitope) might mean. It is readily apparent that there are multiple reasonable senses of such mimicry (*Greenspan*, 1992b). Perhaps the most obvious form of mimicry of one molecule by another is on the level of purely structural resemblance. Two molecules are distinguishable by a chemist, and will have different names, as long as they are non-identical in composition or connectivity, even if the difference reduces to a single atom or covalent bond. It should not be surprising that any two such related molecules, referred to by different names, could share extensive elements of structure. However, even in the case of relatively unrelated molecules with respect to atomic composition, there is the formal possibility of regions of structure that are similar in three-dimensional distribution of electron density. Such similarity could arise from the identical atoms residing at more or less exactly the same relative positions or even from non-identical atoms occupying more or less equivalent relative positions in space over some patch of molecular surface. In this case, the mimicry would likely be limited to one region or face of each of the molecules being compared. Henceforth, the molecule being mimicked will be referred to as the model and the molecule exhibiting the mimicry will be referred to as the mimic.

A second conceivable form of mimicry might be termed immunochemical. This form of mimicry is concerned with non-covalent binding. The tendency to presume a strong correlation between structural and immunochemical mimicry should be resisted, not because such a correlation does not exist, but because the correlation is not reliable. Non-covalent interactions between biological macromolecules or between macromolecules and small ligands involve multiple factors, as noted above, and the most general approach presumes as little as possible. Once a given molecule, the mimic, is found to bind to any number of receptor molecules similarly to another molecule, the model, it may be of interest to know what structural relationship between mimic and model accounts for such similarity in binding. In some cases, it will be found that the structural similarities are not as impressive as some might have anticipated.

Consider the case of insulin, as reported by *Weiss* and colleagues (*Hua* et al., 1991). They note that there is a mutant form of insulin differing from the wild-type amino acid sequence by a single amino acid substitution. Furthermore, the crystallographic structure of

this mutant form of insulin is, with the exception of the one altered side chain, virtually identical to that of the wild-type form. Nevertheless, this molecule is not functional. In contrast, *Hua* et al. (1991) describe a point mutant of insulin that retains insulin function despite having a significantly different structure than the wild-type molecule by nuclear magnetic resonance (NMR) spectroscopy. Thus, this set of insulin molecules provides a compelling example of the potential for divergence between structural similarity and functional similarity.

The third form of mimicry that will be of strong interest to biomedical investigators is functional mimicry. In this instance, a biological response is elicited following non-covalent interaction between either the model or the mimic and, most typically, a macromolecular receptor of some sort. As in the case of immunochemical mimicry, this form of mimicry can be evaluated on its own operational basis, and it is best not to presume that a given degree of functional similarity necessarily reflects any given magnitude of structural or immunochemical mimicry.

It is not difficult to imagine molecules that elicit similar functional responses from different receptors on the same or different cells, or even different responses from the same receptor under different cellular or environmental circumstances. For example, binding to T cell receptors can have distinct consequences for immature T lineage cells in the thymus in comparison to mature T lymphocytes in the periphery. Another interesting example is provided by a study (*Tran Van Nhieu* and *Isberg*, 1993) of antibodies to a cell-surface integrin able to mediate uptake of attached particles, including the bacterial pathogen *Yersinia pseudotuberculosis*. The authors of this report found that different monoclonal antibodies specific for the integrin, as well as a protein expressed by *Y. pseudotuberculosis*, exhibited the ability to be endocytosed even though they bound to the receptor at distinct sites and therefore, presumably lacked a high degree of structural similarity. Presumably, in this case, the ability to bind to and cross-link the cell-surface integrin, regardless of the location of the contacted site on the integrin, is the basis for the similar functional activities of the molecules active in internalisation. Such degeneracy, in the relationships among different forms of molecular similarity, is also observed in cases where the endpoint is only binding, and not cellular function.

The non-functional insulin mutant, studied by *Hua* et al. and noted above, illustrates a general point worth noting: that, in general, the function of a molecule is not an intrinsic property of the molecule itself. Instead, the function of a molecule, X, is a property that depends on the relationships between X and the molecules with which X interacts. This important principle has also been demonstrated by studies of chimeric cell surface receptors that express the extracellular ligand-binding domain(s) of one molecule and the intracellular signal transducing domain(s) of another molecule. Consider two ligands (e.g., cytokines, growth factors, or hormones) X and Y, and their respective receptors, XR and YR. If the extracellular portion of XR is genetically fused to the intracellular portion of YR, and the chimeric receptor is expressed in a cell line, then it will not be surprising if the function of X with respect to the *X/YR*-expressing cell line (which does not express XR) is more like the function of Y than that of X.

With respect to structure, binding, and the elicitation of higher-order biological function, it is reasonable to determine not only whether there is or is not mimicry of a model molecule by a molecular mimic, but to what extent mimicry is exhibited. Any effort to

quantitate mimicry is likely to reveal that there are multiple ways of precisely defining the extent of mimicry. Consider functional mimicry. Binding to a cell surface receptor, such as an antigen-specific B or T lymphocyte receptor can have multiple consequences, such as proliferation or cytokine secretion. Each endpoint that can be envisioned can be used as the basis for the quantitation of the similarity in functional effects associated with either of the two molecules. There is reason to believe that these endpoints will not always correspond exactly. For example, *Evavold* and *Allen* (1991) demonstrated that changing a single amino acid in a peptide presented to a CD4+ T cell by class II major histocompatibility complex (MHC) molecule can result in activation of the T cell with respect to one function (IL-4 secretion) but not another (proliferation). In other words, the modified peptide is a better mimic of the original peptide for the elicitation of IL-4 secretion than for the elicitation of a proliferative response.

EXPERIMENTAL EVIDENCE PERTAINING TO ANTI-IDIOTYPIC MIMICRY

In this section, selected, but representative, experimental results pertaining to anti-idiotypic mimicry of antigen will be briefly discussed. First, highlights from my own studies on antibodies specific for the group-defining cell wall polysaccharide of group A streptococcus (*Streptococcus pyogenes*) will be summarised. This polysaccharide will be referred to as group A carbohydrate (GAC). Then, insights gained from some of the first crystallographic structures of Id-anti-Id complexes are discussed.

From a large panel of monoclonal antibodies (mAbs; more than 30) secreted by hybridomas and specific for GAC, one mAb (HGAC 39) was chosen to immunise rats for the production of anti-idiotypic antibodies. Nine monoclonal anti-idiotopes specific for the variable module of HGAC 39 were produced and characterised (*Greenspan* and *Davie*, 1985a). Each monoclonal anti-Id exhibited a unique reactivity pattern with the HGAC mAbs. One, anti-IdX, reacted almost equivalently with about half of the panel of GAC-specific mAbs and failed to react detectable with the remaining GAC-specific mAbs. A variety of studies, using different methods, suggested that anti-IdX bound far from the paratope of HGAC 39, and this conclusion was directly confirmed by electron microscopy of negatively stained Id–anti-Id complexes (*Roux* et al., 1987). Such an anti-Id would not be expected to be an impressive mimic of the cell wall polysaccharide epitope. In contrast, another monoclonal anti-Id, anti-IdI-3a, bound to a large fraction of the HGAC mAb panel with varying effectiveness (*Greenspan* and *Davie*, 1985b). The assays that suggested the proximal location of the idiotope recognised by anti-IdX also suggested the distal location of the idiotope recognised by anti-IdI-3a. Electron microscopic analyses supported this conclusion. Since the interaction between HGAC 39 and anti-IdI-3a was directly inhibited by free N-acetyl-glucosamine (GlcNAc), a dominant component of the GAC epitope recognised by HGAC 39, it seemed reasonable to entertain the notion that anti-IdI-3a was a Ab2β, or internal image.

Several results suggest why a dichotomy, that classifies anti-idiotopes as internal images or not, is simplistic. First, while available results suggest

that GAC and anti-IdI-3a mutually compete for binding to overlapping regions of the HGAC 39 variable module, anti-IdI-3a binds to varying degrees to GAC-specific mAbs that all react comparably with GAC. Furthermore, anti-IdI-3a probably binds with considerably higher intrinsic affinity to HGAC 39 than do GlcNAc or GAC. When C57BL/6J mice were immunised with three isotype-matched anti-Ids specific for HGAC 39, anti-IdI-3a elicited the more GAC-specific serum antibodies than the other two anti-Ids or control rat IgG (*Monafo* et al., 1987). Thus, using induction of a primary antibody response to GAC in C57BL/6J mice as the measure of mimicry, anti-IdI-3a was the best mimic among the anti-Ids tested. Interestingly, when primary immunisation of C57BL/6J mice with anti-Id was followed by immunisation with whole heat-killed, pepsin-treated group A streptococci, the anti-Id that was most effective at priming for a secondary response to GAC was anti-IdX. Therefore, under the conditions of this particular immunisation protocol, and with respect to this measure of mimicry, anti-IdX was a better mimic of GAC than anti-IdI-3a, despite the fact that anti-IdX binds to the HGAC 39 at a site distinct, and relatively distant from, the antigen-binding site.

This brief summary of the studies on the antibodies and anti-Ids in the GAC system suggests that mimicry has many measures or dimensions (*Greenspan* and *Bona*, 1993). One of a set of anti-Ids may be the best mimic with respect to one such measure and yet not be the best with respect to another. Furthermore, there is no certain relationship between the extent to which idiotope and paratope overlap and the extent of immunochemical or functional mimicry of antigen by anti-Id *Greenspan* and *Roux*, 1987).

The crystal structures of several complexes between idiotypic and anti-idiotypic Fab or Fv fragments have been studied (e.g., *Bentley* et al., 1990; *Ban* et al., 1994; *Evans* et al., 1994; *Fields* et al., 1995). For the purpose of understanding anti-idiotypic mimicry of antigen, the study of *Fields* and colleagues (1995) is the most relevant, as the anti-Id in this study, E5.2 is able to mimic the antigen, hen egg lysozyme (HEL), in eliciting HEL-reactive antibodies when injected into mice.

The crystal structure of Fv fragments of E5.2 and the HEL-specific mAb, D1.3, reveals many contacts that are similar to those in the D1.3-HEL complex. While the extent of such similarities is impressive, it is equally important to note that the two complexes have numerous differences. For instance, while six hydrogen bonds between the Fv fragments of E5.2 and D1.3 are superimposable on hydrogen bonds of the D1.3-HEL complex, seven are not so superimposable. While 13 residues of D1.3 that contact E5.2 also contact HEL, there are five D1.3 residues that contact E5.2 that do not contact HEL and four D1.3 residues that contact HEL that do not contact E5.2. Thus, even in this most impressive example of structural and functional mimicry, the structural mimicry cannot be considered complete.

CONCLUSION

The notion that anti-Ids can be readily classified into two (or even a few) categories that will correlate with the locations of the corresponding idiotopes on the idiotypic V module is too simple to handle the reality of Id-anti-Id interactions. Such interactions, after all, like other non-covalent interactions, can

vary over a spectrum of affinities and arise from different combinations of atomic level forces. Furthermore, thermodynamic considerations suggest that the affinity and specificity exhibited by a given Id-anti-Id pair are the result of factors that are not obvious solely from detailed analysis of the interface created by formation of the Id-anti-Id complex. Thus, one of a group of anti-Ids may mimic an antigen better than the other anti-Ids with respect to one measure of mimicry and less well with respect to a different measure of mimicry. When the dimensional and quantitative complexities of such interactions are acknowledged, the loss in simplicity is balanced by an increased ability to describe the experimental realities.

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THE NEONATAL INTESTINAL MICROFLORA AND THE IMMUNE SYSTEM

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SUMMARY

Directly after birth, bacteria commence to colonise the skin and the mucous membranes of the respiratory, urogenital and intestinal tract. With time, more or less complex bacterial ecosystems are established at various body surfaces, of which the normal intestinal microflora is the largest and most diverse. Thus, an adult individual harbours more than 400 bacterial species at this site. Most of these bacteria are obligatory anaerobic.

The establishment of the intestinal microflora after birth precedes in a sequential manner, and a complete microflora is not obtained until after several years of age. A number of factors may influence the establishment of the microflora, including delivery and feeding mode, social contacts and the degree of environmental hygiene.

Many bacteria that inhabit the intestine, especially facultatively anaerobic bacteria, are potential pathogens, since they may spread to extra-intestinal sites. High population levels of these bacteria in the intestine in early life make the new-born infant vulnerable to infections, for instance septicaemia. On the other hand, these bacteria may be of great importance for the maturation of the developing immune system. Bacteria colonising the intestine activate both mucosal and systemic immunity, and may contribute to development of oral tolerance towards harmless antigens.

This paper describes the establishment of the intestinal microflora in early life, factors affecting the colonisation process, and influence of the intestinal microflora on the infant's immune system.

ESTABLISHMENT OF THE MAJOR BACTERIAL GROUPS IN THE INTESTINE

The new-born infant is exposed to a wide range of different bacteria, but not all are able to establish and colonise in the neonatal intestine. The implantation of various bacteria into the intestinal microflora is regulated through limitations in the intestinal milieu, which may change with the successive establishment of different bacteria. In summarising what is known of the pattern of colonisation in the neonatal period, it is important to realise that there are considerable variations between studies as to when different bacterial groups settle in the intestine. This likely reflects both differences in methodology as well as study populations.

Aerobic and facultatively anaerobic bacteria

The intestinal milieu is characterised by a positive oxidation-reduction potential during the first days of life (*Grutte* et al., 1965), which favours the growth of aerobic or facultative bacteria, such as *E. coli* and other enterobacteria, enterococci and staphylococci (*Mitsuoka* and *Kaneuchi*, 1977; *Balmer* and *Wharton*, 1989; *Bennet* et al., 1991). These bacteria often reach population levels of 10^{10} bacteria/g faeces in the new-born infant, which is roughly 100 times more than those found in the microflora of adults (*Hoogkamp-Korstanje* et al., 1979; *Stark* and *Lee*, 1982).

E. coli strains that colonise the neonate may derive from the mother's faecal flora, in which case they are often transferred during delivery. Such transfer was frequently observed by *Bettelheim* and co-workers in the seventies (*Bettelheim* et al., 1974), whereas most other studies from Western countries (*Gothefors* et al., 1976; *Fryklund* et al., l992), and even a study of home delivered infants in an urban slum area in Pakistan (*Adlerberth* et al., 1999a) report that only a minority of neonates acquire their dominant intestinal *E. coli* strains from their mothers. It is likely that transfer of bacteria from mother to infant during delivery is markedly reduced when mothers give birth laying on their back and faecal soiling of the infant is avoided, whereas a standing or kneeling delivery position, as practised by certain indigenous populations, almost invariably leads to faecal contamination of the infant and most likely to transfer of large amounts of maternal bacteria to the neonate (*Mata* and *Urrutia*, 1971).

In maternity and neonatal wards, *E. coli* strains may be spread between infants via the nurses' hands (*Bettelheim* and *Lenox-King*, 1976; *Gothefors* et al., 1976). This type of transmission is greatly reduced when "rooming in" is practised, i.e. when the mothers themselves and not the staff handle the babies (*Bettelheim* et al., 1983). Later on, *E. coli* may be acquired from other family members or other persons in contact with the neonate.

Other enterobacteria, such as *Klebsiella*, *Enterobacter* and *Citrobacter*, are isolated from 20-60% of neonates, and in colonised infants reach similar population numbers as *E. coli* (*Lundequist* et al., 1985; *Balmer* and *Wharton*, 1989). These enterobacteria are less common than *E. coli* in the flora of adult individuals, and the strains colonising neonates are therefore rarely of maternal origin. Instead they derive from the intestinal microflora of other neonates, transferred via the staff, or from environmental sources (*Fryklund* et al., 1992; *Adlerberth* et al., 1999a).

Enterococci (*Enterococcus faecalis* or *E. faecium*), are isolated from almost all neonates and commonly reach population levels of 10^{10} CFU/g of faeces (*Rotimi* and *Duerden*, 1981; *Stark* and *Lee*, 1982). Some neonates may acquire these bacteria from their mothers, although this has never been particularly studied. In addition, enterococci are very good at spreading and surviving in the hospital milieu.

Staphylococci, e.g. *Staphylococcus epidermidis* or *S. aureus*, also establish in the intestine of many neonates during the first days of life and may reach population levels of 10^{10} bacteria/g faeces (*Balmer* and *Wharton*, 1989). Although commonly found in low numbers in the intestinal flora of adults, staphylococci are mostly regarded as members of the skin flora, which is probably the origin of many strains colonising the infant.

Aerobic streptococci are also found in the early neonatal microflora (*Rotimi* and *Duerden*, 1981), and strains of the Gram-negative genera *Aeromonas*, *Pseudomonas* and *Acinetobacter* may be transiently isolated from neonates dur-

ing the first week of life (*Rotimi* et al., 1985; *Adlerberth* et al., 1999a).

Anaerobic bacteria

The establishment and expansion of aerobic and facultatively anaerobic bacteria lower the redox-potential to negative values and "makes way" for obligatory anaerobic bacteria (*Hoogkamp-Korstanje* et al., 1979; *Stark* and *Lee*, 1982). Anaerobes recognised as early colonisers include *Bacteroides*, bifidobacteria and clostridia, which may reach populations of 10^{9-11}/g faeces within a week after birth (*Mata* and *Urrutia*, 1971; *Rotimi* and *Duerden*, 1981; *Stark* and *Lee*, 1982).

Infants delivered by caesarean section, who do not come into contact with the maternal faecal, vaginal or perineal flora at delivery, show a delayed colonisation with anaerobic bacteria, especially *Bacteroides* (*Neut* et al., 1987; *Grönlund* et al., 1999). *Bacteroides* die rapidly in contact with environmental oxygen and, since this limits transfer in the hospital milieu, acquisition of *Bacteroides* from the mother is probably of great importance.

Bifidobacteria are considerably more aerotolerant than *Bacteroides*. They are probably spread between neonates in maternity wards, since bifidobacterial colonisation pattern is ward-dependent (*Mitsuoka* and *Kaneuchi*, 1977; *Lundequist* et al., 1985). However, transfer of bifidobacteria from mother to infant also occurs. *Tannock* and coworkers demonstrated bifidobacterial strains with the same ribotype in mother and infant in two out of five mother/infant pairs (*Tannock* et al., 1990).

Clostridia are commonly isolated from the intestinal flora of neonates. These bacteria form spores which are spread in the environment and which are resistant to hygienic measures. In accordance, clostridial species are usually the first anaerobes to colonise infants after sectio deliveries (*Neut* et al., 1987). Strains of e.g. *C. difficile* colonising neonates are almost never acquired from the mother, but are frequently spread between neonates in maternity and neonatal wards (*Martirosian* et al., 1995).

In many recent studies, lactobacilli have been detected in as many as 60-80% of one month old infants (*Hall* et al., 1990; *Kleessen* et al., 1995), whereas such colonisation was infrequently observed in earlier studies (*Mata* and *Urrutia*, 1971; *Ellis-Pegler* et al., 1975; *Stark* and *Lee*, 1982). It is likely that improved bacteriological methods have facilitated the identification of these bacteria, which could have been grouped with e.g. bifidobacteria in earlier studies. Although lactobacilli dominate the vaginal microflora of fertile women, these lactobacilli do not seem to colonise the intestine of the baby (*Tannock* et al., 1990). The mother's faecal flora is a more likely source of infants' intestinal lactobacilli, as the dominating species in adults' intestine, *L. plantarum* (*Ahrné* et al., 1998), dominates also in neonates (*Bennet* and *Nord*, 1987). However, lactobacilli are probably easily acquired also from other sources. Within one month, sectio-delivered infants carry lactobacilli at a rate similar to vaginally delivered infants (*Hall* et al., 1990; *Grönlund* et al., 1999).

It may take a long time before many other intestinal anaerobic bacteria, including *Veillonella*, *Eubacterium*, *Peptostreptococcus*, *Peptococcus* and *Ruminococcus*, establish in the intestine, although there are large variations between different studies (*Mata* and *Urrutia*, 1971; *Ellis-Pegler* et al., 1975; *Mitsuoka* and *Kaneuchi*, 1977; *Rotimi* and *Duerden*, 1981; *Stark* and *Lee*, 1982; *Benno* et al., 1984; *Lundequist* et al., 1985; *Kleessen* et al., 1995; *Sepp* et al., 1997).

Many of the strict anaerobic bacteria

colonising the intestine can not be cultured *in vitro*. Little is known about these bacteria and their time of establishment in the microflora. In mice, segmented filamentous bacteria, a group of non-culturable, strictly anaerobic, spore-forming, Gram-positive bacteria (*Klaasen* et al., 1992), colonise the intestinal tract and become the dominating microbes around the time of weaning (*Garland* et al., 1982). Related bacteria occur also in humans (*Klaasen* et al., 1992).

When the anaerobic bacterial populations expand in the intestine, facultative bacteria are suppressed and decline in numbers (*Mata* and *Urrutia*, 1971; *Stark* and *Lee*, 1982). Within a few weeks or months, reduced counts of e. g. *Klebsiella*, *Enterobacter* and staphylococci are observed (*Ellis-Pegler* et al., 1975; *Rotimi* and *Duerden*, 1981; *Balmer* and *Wharton*, 1989; *Kleessen* et al., 1995; *Adlerberth* et al., 1999a). Other facultative bacteria, such as *E. coli* and enterococci retain quite high population numbers for longer periods of time (*Bennet*, 1987). Thus, high levels of both facultatives and anaerobes may co-exist during the first months (*Hoogkamp-Korstanje* et al., 1979; *Stark* and *Lee*, 1982), or even years of life (*Ellis-Pegler* et al., 1975). This relates to the fact that a diversified anaerobic microflora is required to suppress e.g. the numbers of *E. coli* in the intestine. In mice mono-associated with *E. coli*, population levels of $10^{10\text{-}11}$ bacteria/g faeces are obtained. To bring down the *E. coli* population to the levels found in conventional animals, 95 different anaerobic strains isolated from conventional mice are required (*Freter*, 1992). In humans, the successive establishment of different anaerobic species into the intestinal microflora proceeds over a period of several years (*Ellis-Pegler* et al., 1975; *Midtvedt*, 1994), finally resulting in a pronounced anaerobic predominance. The ratio of anaerobes to aerobes has been calculated to be 1.5 before 4 month's age, 10 between 4 and 12 month's age, 50 between 1 and 4 year's age and 200 in an adult (*Ellis-Pegler* et al., 1975).

THE INFLUENCE OF BREASTFEEDING ON THE INTESTINAL MICROFLORA

A great number of studies have investigated the intestinal microflora in breastfed and bottlefed infants. The results vary considerably between studies, but some differences between breastfed and bottlefed infants are quite consistently observed. Thus, most studies find lower counts of clostridia and enterococci in breastfed than in bottlefed infants (*Stark* and *Lee*, 1982; *Benno* et al., 1984; *Lundequist* et al., 1985; *Balmer* and *Wharton*, 1989; *Kleessen* et al., 1995), whereas breastfed infants tend to have higher counts of staphylococci than bottlefed infants, especially during the neonatal period (*Lundequist* et al., 1985; *Balmer* and *Wharton*, 1989). The reason may be that staphylococci colonising the nipple are swallowed during breastfeeding (*Gothefors*, 1975).

High bifidobacterial counts were a long time regarded as the most characteristic feature of the intestinal flora of the breastfed infant (*Bullen* et al., 1976). However, most studies from the eighties and onwards report similar counts of bifidobacteria in breastfed and bottlefed infants (*Lundequist* et al., 1985; *Balmer* and *Wharton*, 1989; *Balmer* et al., 1994; *Kleessen* et al., 1995). Nevertheless, more breastfed than bottlefed infants may harbour a flora dominated by bifidobacteria, most-

ly due to lower levels of other bacterial groups (*Balmer* and *Wharton*, 1989). Lactobacilli, which is another group of acid tolerant bacteria, are not favoured in the intestine of breastfed babies (*Benno* et al., 1984; *Kleessen* et al., 1995).

Only a minority of studies report higher counts of *Bacteroides* or enterobacteria in bottlefed than in breastfed infants (*Bullen* et al., 1976; *Benno* et al., 1984; *Balmer* and *Wharton*, 1989). However, the composition of the enterobacterial flora differs at the species and strain level between breastfed and bottlefed infants. Breastfed infants less often carry enterobacteria other than *E. coli*, for example *Klebsiella* or *Enterobacter* (*Örskov* and *Biering-Sörensen*, 1975; *Bullen* et al., 1976; *Balmer* and *Wharton*, 1989; *Adlerberth* et al., 1991). They also have a more stable enterobacterial flora than bottlefed infants, with fewer different *E. coli* serotypes being present concomitantly (*Örskov* and *Biering-Sörensen*, 1975; *Mevissen-Verhage* et al., 1985) and over time (*Mevissen-Verhage* et al., 1985). It seems reasonable to assume that differences at the species and strain level between breastfed and bottlefed infants may exist also for other major bacterial groups in the intestinal microflora.

A number of different factors in breastmilk has been suggested to contribute to differences in the bacterial flora between breastfed and bottlefed infants. These include the low buffering capacity of human milk (*Bullen* et al., 1976), and factors like secretory IgA, lactoferrin, lysozyme, complex oligosaccharides and nucleotides (*Wold* and *Hanson*, 1994). However, feeding with formulas supplemented with e.g. lactoferrin or nucleotides or formulas with reduced buffering capacity have not resulted in a "breastfed" type of microflora in the infants studied (*Balmer* et al., 1989a; 1989b; 1994).

GLOBAL DIFFERENCES IN INTESTINAL COLONISATION PATTERN - IMPACT OF HYGIENIC CONDITIONS

The intestinal colonisation pattern is strongly influenced by the degree of bacterial exposure and, thus, varies considerably between infants in developing and industrialised societies.

In general, infants born in poor areas in developing countries are earlier colonised with a number of different bacteria than infants in rich and highly developed societies. In indigenous Guatemalan infants, meconium passed at 4-7 hours after birth often contained bacteria, most commonly enterobacteria and streptococci (*Mata* and *Urrutia*, 1971). The mothers gave birth in a kneeling position, and maternal faeces commonly contaminated the infant during delivery (*Mata* and *Urrutia*, 1971), facilitating direct transfer of bacteria from mother to infant. However, under poor hygienic conditions, sectio-delivered neonates as well acquire bacteria almost immediately after birth (*Rotimi* et al., 1985; *Adlerberth* et al., 1991), reflecting heavy environmental exposure to bacteria. In Pakistan, infants from underprivileged groups, whether delivered in hospital or at home and regardless of delivery mode, acquire enterobacteria earlier and harbour a more diverse enterobacterial flora than Swedish infants (*Adlerberth* et al., 1991; 1999a). In an ongoing study, we have observed than at least 40% of Swedish neonates have not yet acquired any enterobacteria at one week of age, and that it takes a month before all infants harbour enterobacteria in their faecal flora (*Adlerberth* et al., 1999b). This shows the severely restricted circulation of enterobacteria in

a highly hygienic modern society.

Pronounced environmental exposure to enterobacteria also leads to a high turn over of enterobacterial strains in the microflora. Thus, different *E. coli* strains replace each other in rapid succession in the intestinal flora of Pakistani infants (*Adlerberth* et al., 1999a) whereas in Swedish infants a single *E. coli* strain usually dominates the enterobacterial microflora for prolonged periods of time (*Kuhn* et al., 1986).

Colonisation with many other bacterial groups, e.g. enterococci, lactobacilli and eubacteria is similarly delayed in Western infants (*Bennet* et al., 1991; *Sepp* et al., 1997). The fact that more Swedish than Estonian one year old infants carry *C. difficile* (*Sepp* et al., 1997) indicate that Swedish infants have a poorly developed intestinal microflora by that age, as *C. difficile* is common in the intestinal microflora of young infants, but usually disappear when a complex anaerobic microflora is established.

In the absence of competition from e.g. enterobacteria, intestinal colonisation with "skin bacteria" like *S. epidermidis* have become more prominent among neonates in Western societies. Thus, early colonisation with *S. epidermidis* is more common in Sweden than in Ethiopia (*Bennet* et al., 1991). Similarly, in French neonates (*Borderon* et al., 1996), *S. epidermidis* rather than *E. coli* or enterococci are the first bacteria to colonise the intestine. Today, all Swedish infants seem to be colonised with staphylococci within three days after birth (*Adlerberth* et al., 1999b).

BACTERIAL TRANSLOCATION

Certain bacteria that colonise the intestinal tract have the capacity to translocate, i.e. pass viable over the intestinal barrier to reach mesenteric lymph nodes, blood or other organs (*Berg*, 1983b). Translocating bacteria include *E. coli* and other enterobacteria, staphylococci, enterococci and lactobacilli. Most obligate anaerobes seem unable to translocate - most likely they do not survive in the oxygen-rich milieu of viable tissues. Translocation may occur when bacteria with the capacity to translocate reach high population levels in the intestine, i.e. more than 10^8 bacteria/g faeces (*Berg*, 1995), as occur e.g. during treatment with antibiotics (*Berg*, 1983b). It is further promoted by deficiencies in the host immune system and damage to the intestinal mucosal barrier (*Berg*, 1995). Although mostly studied in experimental animals, bacterial translocation has been shown to occur also in humans (*Brooks* et al., 1993), and has been suggested to be the mechanism behind septicaemia due to enteric bacteria in immunocompromised patients (*Tancrede*, 1992). Furthermore, bacterial translocation may explain the propensity of enterobacteria to cause septicaemia in new-born, especially premature infants (*Van Camp* et al., 1994). As discussed above, facultative bacteria reach high population levels in the intestinal flora during the neonatal period which, in combination with immature immune functions, may predispose for bacterial translocation and subsequent septicaemia (*Van Camp* et al., 1994). A considerable frequency of asymptomatic bacteraemia, occurring at the time for intestinal colonisation, has been observed in neonates (*Albers* et al., 1966).

Neonatal septicaemia is especially common in developing countries (*Dawodu* and *Alausa*, 1980; *Khan* et al., 1993), where intestinal bacteria such as *E. coli*, *Klebsiella* and other enterobacteria, enterococci and *Pseudo-*

monas are responsible for up to 80% of the cases (*Dawodu* and *Alausa*, 1980; *Bhutta* et al., 1991).

The mechanisms behind bacterial translocation are only partly understood. It is likely that bacteria are taken up via the Peyer's patches and other lymphoid follicles lining the intestinal tract, in which case translocation may be regarded as a physiological process related to the sampling of lumenal contents by the gut immune system, causing disease only if the host defence systems are overridden. In addition, enterocytes under certain conditions may permit passage of live bacteria.

THE NEONATAL IMMUNE SYSTEM

The new-born infant is more susceptible to infections than older children and adults because certain functions of the immune system are to a greater or lesser extent immature in the neonate.

Some aspects of innate immunity are not fully developed at birth. Concentrations of the complement factors C8 and C9 only reach 20 and 10%, respectively, of adult levels (*Ballow* et al., 1974), and the plasma levels of C1, C2, C3, C4, C6 and C7 are also low in the new-born infant (*Fireman* et al., 1965; *Adinolfi* and *Beck*, 1976). In accordance, the sera of neonates show decreased bactericidal activity (*Lassiter* et al., 1992).

The number of phagocytes in the blood are as high in infants as in adults, but the total pool of granulocytes that can be mobilised during infection is small. Neutrophils show reduced adherence and chemotaxis and lower enzymatic activity than in adults (*Klein* et al., 1977; *Kovarik* and *Siegrist*, 1998). Chemotactic activity of monocytes may be impaired, and there is a clearly diminished influx of monocytes into sites of inflammation (*Wilson* et al. 1996). Natural killer cells are present in normal numbers at birth, but show decreased cytotoxic activity compared with cells from adults (*Wilson* et al., 1996).

The specific immune system is fairly well developed at birth. However, although the neonate has the capacity to mount a specific immune response to most antigens, a primary immune response has to take place for each new antigen that is encountered. Thus, neonatal T cells are almost entirely of the naive phenotype (*Lewis* et al., 1991), which means that they are more difficult to activate than memory cells (*Wilson* et al., 1996). Like naive T cells from adults, neonatal T lymphocytes produce high levels of IL-2 when stimulated with mitogens, but very little IFN-γ and IL-4 (*Bodeker* et al., 1982; *Lewis* et al., 1991). They also show an inability to express the CD40 ligand following activation (*Nonoyama* et al., 1995), which limits their capacity to deliver help to B cells (*Wilson* et al., 1996).

The cytotoxic capacity of sensitised T cells, defined as the capacity to lyse allogeneic lymphocytes, is not fully developed in term neonates (*Granberg* and *Hirvonen*, 1980). At least partly, this could reflect the absence of memory CD8+ T cells in neonates, as such cells kill more efficiently than naive CD8+ T cells (*McFarland* et al., 1992; *Wilson* et al., 1996).

Neonatal B cells function is fully mature at birth with regard to IgM formation but not with regard to IgG or IgA formation (*Andersson* et al., 1981). Thus, B cell activation *in vivo* in response to antigens mainly results in IgM production (*Gathings* et al., 1977). This is most likely at least partly related to the inability of neonatal T cells to provide efficient help for B cell differentiation and isotype switch, as discussed above. Neonatal B cells respond poorly to bac-

terial polysaccharides, which are antigens not requiring contact-dependent T cell help (*Wilson* et al., 1996). However, the limited capacity of neonatal T cells to produce IFN-γ could also be of importance in this matter (*Wilson* et al., 1996), as IFN-γ enhances the B cell response to bacterial capsular polysaccharides (*Peeters* et al., 1992).

Another specific feature of the foetal and neonatal B-cell repertoire is the relative preponderance of B cells expressing CD5 (*Bhat* et al., 1992). B cells of the CD5+ or B1 phenotype represent a separate lineage, precursors for which are found only early during development (*Herzenberg* et al., 1986). They typically produce polyspecific antibodies, most often of the IgM isotype, which are often reactive with self-antigens as well as bacterial antigens (*Casali* and *Notkins*, 1989; *Barbouche* et al., 1992). These antibodies may be produced independently of exogenous antigen stimulation, and are thought to play a role in regulation and development of the immune system in early ontogeny and also to act as a first line of defence against invading micro-organisms, before specific immune responses have evolved (*Casali* and *Notkins*, 1989; *Avrameas*, 1991).

The serum levels of immunoglobulin, except IgG, are low in neonates (*Allansmith* et al., 1968). Due to the active transport of IgG antibodies over the placenta, IgG levels in cord blood exceed those found in maternal blood (*Allansmith* et al., 1968). IgG synthesised by the baby is present only in low amounts (*Martensson* and *Fudenberg*, 1965). Immunoglobulins of the IgA class may also be found in cord blood, in concentrations of less than 50 μg/ml (*Allansmith* et al., 1968; *Wilson* et al., 1996). IgM is present in sera of all healthy neonates, in concentrations of 110-150 μg/ml, which resemble approximately 10% of adult levels (*Allansmith* et al., 1968; *Wilson* et al., 1996). Much of the IgM antibodies present at birth are likely to be polyspecific antibodies, produced by CD5+ B cells in the absence of exogenous antigenic stimulation.

THE MUCOSAL IMMUNE SYSTEM IN THE NEONATE

The gut associated immune system contains the vast majority of all lymphoid cells in the human body. This include lymphoid cells dispersed in the lamina propria, intraepithelial lymphocytes, and organised lymphoid tissue, such as Peyer's patches and colonic lymphoid follicles as well as the mesenteric lymph nodes that drain the intestinal tract. At birth, lymph nodes and Peyer's patches contain only primary follicles with mainly IgM+ and IgD+ cells, but very few IgA+ cells (*Russell* et al., 1990). The lamina propria contains very few immunoglobulin-containing cells (*Perkkiö* and *Savilathi*, 1980; *Russell* et al., 1990), which are mainly IgM+ and almost never IgA+ (*Iwase* et al., 1987; *Russell* et al., 1990). In accordance, no or only small amounts of secretory IgA are detected in foetal gut content or in meconium (*Rule* et al., 1971; *Petit* et al., 1973).

CD4+, CD3+ and CD8+ cells are readily identified in the lamina propria of both foetal and neonatal intestine. Many of the CD4+ cells have the morphological appearance of macrophages or dendritic cells (*Russell* et al., 1990). Very few intraepithelial lymphocytes are present at the time of birth, and no epithelial MHC class II expression is observed (*Russell* et al., 1990).

DEVELOPMENT OF THE IMMUNE SYSTEM AFTER BIRTH - RELATION TO INTESTINAL COLONISATION

Systemic immunity

In healthy neonates, there is a rapid increase in serum IgM during the very first weeks after birth (*Allansmith* et al., 1968). Thereafter the levels increase more slowly and 60-100% of adult levels are achieved at one year of age (*Stiehm* and *Fudenberg*, 1966; *Allansmith* et al., 1968). IgG production in the neonate is most likely initiated during the first weeks of life (*Allansmith* et al., 1968). By two months of age, the amount of circulating IgG synthesised by the infant equals the amount derived from transplacental transfer, and by one year of age, almost all circulating IgG is synthesised by the infant (*Wilson* et al., 1996). Sixty to 80% of adult IgG levels are reached by one year's age (*Allansmith* et al., 1968; *Stiehm* and *Fudenberg*, 1966) and adult levels by 7 years' age (*Allansmith* et al., 1968). Of the IgG subclasses, the production of IgG2 and IgG4 increase more slowly than that of IgG1 and IgG3 (*Morell* et al., 1972). The levels of IgA in serum also increase gradually after birth. At one year of age, serum levels of IgA are approximately 20-25% of adult levels, which are not achieved before 12 years of age (*Stiehm* and *Fudenberg*, 1966; *Allansmith* et al., 1968). The most pronounced increase in serum IgA levels is observed during the first 3 months after birth (*Stiehm* and *Fudenberg*, 1966).

The initiation of IgG and IgA production after birth is likely to be a response to bacterial colonisation of the gastrointestinal tract and other mucosae. The importance of the intestinal microflora as a stimulus for antibody production is illustrated by the fact that serum immunoglobulin levels in germfree animals are only 10 to 20% of those in conventional animals (*Sell* and *Fahey*, 1964; *Kim* et al., 1966; *Wostmann* et al., 1971). When germfree animals are colonised by an intestinal flora, immunoglobulin concentrations in serum and secretions rise and antibodies appear towards the colonising micro-organisms (*Carter* and *Pollard*, 1971). In comparison with the strong stimulus afforded by the microflora, the diet contributes very little antigenic stimulation, despite the fact that bacterial components are present in standard animal feed (*Midtvedt* and *Gustafsson*, 1981). Thus, serum IgA and IgG are slightly increased in germfree mice fed a commercial diet compared with those fed an antigen-free diet, but much lower than in conventional mice (*Hashimoto* et al., 1978).

It is less clear how serum IgM levels depend on exogenous antigenic stimulation. A high proportion of the IgM in serum may represent "natural" antibodies, which may be formed as a consequence of endogenous antigenic stimulation by self antigens or antibodies of maternal origin (*Berg*, 1983a). Thus, germfree mice fed an antigen free diet possess normal levels of serum IgM (*Hooijkaas* et al., 1984). However, the very rapid increase in serum IgM observed in human infants during the first weeks of life is most likely a response to antigenic, probably microbial stimulation after birth (*Allansmith* et al., 1968).

Mucosal immunity

Secretory IgA responses develop earlier and independently of serum IgA antibody responses (*South*, 1971). Neonatal secretions contain no or only low levels of secretory IgA (*Rule* et al., 1971; *Petit* et al., 1973; *Burgio* et al., 1980; *Gleeson* et al., 1982; *Mellander* et al., 1984) but relatively more IgM than in older children (*Gleeson* et al., 1982; *Mellander* et al., 1984). This is due to the fact that IgM, in the absence of

dimeric IgA, can bind to secretory component and be transported out into mucosal secretions (*Hanson* et al., 1999).

During the first weeks or months of life, there is a marked increase in secretory IgA in saliva, where-after the levels may decrease slightly and then remain fairly constant for years (*Gleeson* et al., 1982). Adult levels of secretory IgA are reached at the age of 6-8 years (*Burgio* et al., 1980).

Immunohistochemical examinations of human postnatal intestine show the appearance of secondary lymphoid follicles, increasing numbers of IgA positive cells in the lamina propria, increasing numbers of intra-epithelial lymphocytes and the expression of MHC class II antigen on enterocytes (*Perkkiö* and *Savilathi*, 1980; *Russell* et al., 1990; *Rognum* et al., 1992; *Machado* et al., 1994). Expansion of mucosal dendritic cells also occurs postnatally (*MacDonald*, 1996). Levels of soluble IL-2 receptors, secreted by activated T cells and macrophages (*Rubin* and *Nelson*, 1990), rise within days after birth presumably reflecting mucosal immune responses (*Spear* et al., 1995).

The appearance of IgA positive cells in the lamina propria and the rapid increase in secretory IgA levels after birth is most likely primarily the result of colonisation of mucosal surfaces by commensal bacteria. Germfree animals have approximately one tenth as many IgA-producing cells in the intestinal lamina propria as conventional animals (*Crabbé* et al., 1968; *Hashimoto* et al., 1978). *Koopman* et al. (1982) demonstrated an increase in IgA-producing cells in the ileal mucosa and Peyer's patches after associating germfree mice with an intestinal microflora. In contrast, food antigens are very poor inducers of secretory IgA production (*Wold* et al., 1989).

Animal studies have shown that also the presence and activation state of intra-epithelial lymphocytes as well as the expression of MHC class II molecules on enterocytes are strongly dependent on the presence of a normal microflora (*Umesaki* et al., 1993; 1995). In response to lumenal bacteria, intra-epithelial lymphocytes start to produce IFN-γ, which in turn upregulates the expression of MHC class II molecules on intestinal epithelial cells (*Matsumoto* et al., 1999).

In humans, 24-74% of intestinal bacteria are coated with IgA *in vivo* (*van der Waaij* et al., 1996). The capacity of a bacterial strain to induce strong immunity is linked to its ability to colonise and to invade the Peyer's patches (*Hohmann* et al., 1979). When a bacterial strain successfully colonises the intestine and reaches numbers high enough to permit translocation, a stimulation of the Peyer's patches resulting in the formation of germinal centres occurs. Activated B cells leave the patches and home to the lamina propria where they differentiate into IgA-producing plasma cells. The secretory IgA so produced coats the bacteria in the intestinal lumen, which most likely prevents further translocation. Thus, despite the continued presence of the microbe in the gut flora, there will be no, or only minimal, further stimulation of the immune system (*Shroff* et al., 1995). Therefore, it is likely that a persistent activation of the mucosal immune system depends on the continuous acquisition of new bacterial strains in the microflora.

Only a limited portion of the secretory IgA produced in response to a microbe colonising the intestine seem to be specific for this microbe (*Cebra*, 1999). A significant proportion of the secretory IgA produced may, in fact, represent polyreactive antibodies (*Vassilev* and *Veleva*, 1996; *Quan* et al., 1997). It is possible that a portion of intestinal lamina propria IgA plasma cells are derived from B1 cells residing in the peritoneal cavity (*Kroese* et al., 1995). In addition, it has been shown that IgA secret-

ing hybridoma clones obtained from mouse Peyer's patches may also produce polyreactive antibodies (*Shimoda* et al., 1999). Polyreactive antibodies could possibly act as a first line of defence at mucosal surfaces, before the induction of specific immune responses (*Quan* et al., 1997).

The pattern of intestinal colonisation may influence the "maturation" of the immune system

As mentioned above, it is likely that a persistent activation of the mucosal immune system requires a high turn-over of bacterial strains in the intestinal microflora. Pakistani infants, who are early colonised and constantly acquire new enterobacterial strains in their intestinal flora, have higher secretory IgA levels in saliva, and higher anti-*E. coli* antibody levels than Swedish infants of the same age (*Mellander* et al., 1985). Also, *Nagao* and co-workers (1993) found that children living in slum areas in Sao Paulo had higher levels of salivary IgA than Brazilian middle-class children, most likely reflecting differences in microbial exposure between the groups.

Breastfeeding, the intestinal microflora and the immune system

Breastfed infants may experience less translocation, partly because they are colonised with fewer different strains, but mainly because secretory IgA, which is present in high concentrations in the milk, directly prevents translocation of gut bacteria (*Maxson* et al., 1995). A reduction of translocation may be a major reason for the strong protection against sepsis afforded by breastfeeding (*Winberg* and *Wessner*, 1971; *Ashraf* et al., 1991). Thus, it is likely that breastfeeding reduces the load of different microbial antigens reaching the intestinal immune system. Accordingly, immunocompetent mouse pups nursed by SCID/SCID mothers, and therefore are not supplemented with IgA via the milk, undergo an accelerated development of IgA responses (*Kramer* and *Cebra*, 1995; *Cebra*, 1999).

Many prospective studies show a more rapid and prominent increase of salivary IgA after birth in bottlefed than in breastfed infants (*Stephens*, 1986; *Gleeson* et al., 1986). Serum IgA responses may also be elevated in bottlefed neonates (*Sarrinen* et al., 1979), and *Stephens* et al. (1984) demonstrated significantly higher levels of IgM anti-*E. coli* antibodies in bottlefed compared to breastfed babies from the sixth day of life. The baseline activation of lymphocytes also seems to be higher in bottlefed than in breastfed infants, as reflected by a higher integrin expression and pronounced proliferative responses in the absence of antigen (*Pabst* et al., 1997). The antibody response to mucosal vaccines (e.g. live poliovirus and rotavirus) is often lower in breastfed than in bottlefed infants, which is generally attributed to an inhibition of virus replication in the gut or an enhanced clearance of virus from mucosal surfaces due to antiviral secretory IgA antibodies and other factors in breastmilk (*Pichichero*, 1990; *Rennels*, 1996).

However, breastmilk contains a host of factors that might modulate the developing immune system (*Wold* and *Hanson*, 1994). Large amounts of both inflammatory (IL-1, TNF-α) and anti-inflammatory (TGF-β, IL-10) cytokines are present in breastmilk (*Wold* and *Hanson*, 1994). Animal experiments show that cytokines survive and retain biologic activity during the passage through the gastrointestinal tract and that they may even be taken up into the circulation and thus affect immune functions (*Rollwagen* and *Baqar*, 1996). Breastmilk also contains large numbers of macrophages and activated T lymphocytes (*Wold* and *Hanson*, 1994) which could also influence the infant's immune system. Indeed, radiolabelled

human breastmilk leukocytes fed to new-born baboons are taken up into the circulation (*Jain* et al., 1989). The transient tuberculin positivity observed in breastfed infants born to tuberculin-positive mothers provides indirect evidence that functionally active T cells are taken up from the maternal milk by the infant (*Schlesinger* and *Covelli*, 1977). Furthermore, a range of hormones, such as thyroxin, insulin and corticosteroids, and growth factors, e.g. epidermal growth factor, nerve growth factor and insulin-like growth factor, are present in human milk (*Koldovsky* and *Thornburg*, 1987). Human milk also contains considerable amounts of mononucleotides, which may augment proliferative responses by lymphocytes (*Carver* et al., 1990). Thus, although reducing antigenic stimulation, breastfeeding may have other stimulating effects on the neonatal immune system.

The intestinal microflora and oral tolerance

Food proteins and other harmless soluble antigens usually induce a state of specific unresponsiveness, termed oral tolerance, when presented to the immune system via the oral route (*Telemo* et al., 1997). To uphold a state of tolerance to these antigens is an important task of the immune system; in the absence of such mechanisms inflammatory and hypersensitivity reactions might occur.

The presence of a normal bacterial flora in the gut facilitates the induction of oral tolerance to food antigens (*Moreau* and *Corthier*, 1988; *Sudo* et al., 1997). Further, the administration of LPS together with food antigens increases the tolerising effect of feeding (*Kim* and *Ohsawa*, 1995). The mechanisms behind these interactions have not been defined but could possibly involve effects of the intestinal microflora on antigen presenting cells (*Wold* et al., 1998). Several factors secreted by macrophages in response to bacterial products have been shown to decrease the antigen presenting capacity of dendritic cells (*Holt* et al., 1993; *Chouaib* et al., 1985), which are the cells likely to present soluble protein antigens to T cells (*Steinman*, 1991; *Wold* et al., 1998). A downregulation of the antigen presenting capacity of dendritic cells could be crucial for the induction of oral tolerance to soluble protein antigens (*Wold* et al., 1998).

It is possible that the early establishment of high population levels of certain bacteria in the neonatal intestine provides stimulus for the developing immune system of importance for the induction of tolerance to e.g. food proteins and inhaled environmental antigens. Thus, in Western societies, where colonisation with certain bacterial groups is delayed and the intestinal microflora of infants seem to be less diverse than in developing countries, too little stimulation of the immune system could hamper tolerance induction (*Sepp* et al., 1997; *Wold* et al., 1998). The incidence of atopic allergy is steadily increasing in Western countries (*Björkstén*, 1994). An interesting speculation is that the high incidence of allergy relates to an inadequately developed intestinal microflora of infants in these societies (*Sepp* et al., 1997; *Wold* et al., 1998).

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INNATE AND SPECIFIC MUCOSAL IMMUNITY

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SUMMARY

A large mucosal immune system controlled by unique, organ-specific regulations operates alongside and separate from the peripheral or systemic immune system. This local immune system is seen as a major interface between the innate and the specific immune system. We have been interested in the immunopathogenesis of a chronic inflammatory bowel disease (IBD) developing in $CD4^+$ T cell-transplanted, immunodeficient (SCID) mice. Microbial-derived factors drive (directly or indirectly) the activation, expansion and/or Th1 differentiation of adoptively transferred $TCR\alpha\beta$ $CD4^+$ T cells in the colonic lamina propria of transplanted, diseased SCID mice with colitis (the particular manifestation of IBD observed in this model). A TCR-independent, polyclonal stimulus seems to be the major stimulus for this cellular immune response in the colon. This suggests that T cells of the specific peripheral immune system that migrate into compartments of the mucosal micro-environment change their activation requirements and acquire responsiveness to stimuli that usually drive the innate immune system.

INTRODUCTION

The mucosal surface area of the airways and the intestine is more than two orders of magnitude larger than the surface area of the skin comprising an estimated 400 m^2. It is continuously and extensively exposed to antigens derived from either (resident or invading) micro-organisms, or ingested or inhaled foreign material. This antigenic challenge operates in an environment in which bacterial products with potent adjuvant activity are continuously and abundantly present. Furthermore, the mucosal immune system is the portal of entry of most pathogenic micro-organisms that makes it the focus of interest of research on infectious disease immunity and vaccine designs.

Abbreviations used:
IBD: inflammatory bowel disease; SCID: severe combined immunodeficiency; IEL: intraepithelial lymphocyte; LPL: lamina propria lymphocyte; mLN: mesenteric lymph node; NK: natural killer; CTL: cytotoxic T lymphocyte; Th: T helper cell; PP: Peyer's patch; GALT: gut-associated lymphoid tissue; CP: cryptopatch; IEC: intestinal epithelial cell; APC: antigen-presenting cell; DC: dendritic cell; UC: ulcerative colitis; CD: Crohn's disease; TNBS: tri-nitro-benzene-sulfonic acid; TNP: tri-nitro-phenyl-; SPF: standard pathogen-free; GF: germ-free; AICD: activation-induced cell death; R: receptor; IL: interleukin; LT: lymphotoxin; f: formylated; pIgA: polymeric IgA; PSGL-1: P selectin-glycoprotein ligand-1; KO: knock-out; TCR: T cell receptor for antigen; FCM: flow cytometry; ELISA: enzyme-linked immunoabsorbant assay; Tr1: regulatory T cell subset 1; $IFN\gamma$: interferon-γ; $TNF\alpha$: tumor necrosis factor-α.

To cope with this massive and continuous challenge with potent antigens and adjuvants of a very heterogeneous nature, the mucosa has evolved a large organ-specific immune system. This finely regulated mucosal immune system exists alongside and separate from the peripheral or systemic immune system. It is very large containing about 80% of all T cells of the organism. Mucosal immunity is made up of heterogeneous components that mediate innate and adaptive (specific), humoral and cellular immune reactivities. Priming this system results in local immunity, in generalised immunity on many different mucous surfaces, in systemic immunity, and in immunity in solid organs (as e.g. liver and pancreas). In addition to inducing immunity, a predominant mode of reaction of the mucosal immune system is the induction of tolerance.

Although the mucosal immune system is highly integrated and finely regulated, dysregulation of the induction and regulation of mucosal responses are not unusual. The system can be either deficient to react, or prone to overreact. Many pathogens overcome the mucosal immune barrier and establish themselves transiently or permanently in the host. Furthermore, mucosal tissues are very susceptible to damage by dysregulated immune responses resulting in chronic inflammatory processes.

Hence, we are looking at a defence system that is continuously challenged by a high load of antigens and adjuvants, that has evolved many (humoral and cellular) components to react, but is prone to dysregulation of its responsiveness.

FEATURES OF THE MUCOSAL IMMUNE SYSTEM

The mucosal immune system displays many unique features. Only some general characteristics are mentioned below.

Lymphopoietic, inductive and effector compartments co-exist within the mucosa

Mucosa-associated lymphopoiesis is evident in clusters of about 10^3 cells in the lamina propria of the small intestine situated just under the crypt epithelium and designated cryptopatches (CP) (*Hurst* et al., 1997; *Saito* et al., 1998; *Page* et al., 1998; *Howie* et al., 1998). These areas contain progenitor cells for T cells of the TCR $\alpha\beta$ and $\gamma\delta$ lineage (but not B cells or myeloid cells) with the phenotype $ckit^+$ Lin^- $IL\text{-}7R^+$ $Thy\text{-}1^+$ $CD44^+$ $RAG\text{-}1/2^+$ that differentiate under the influence of IL-7 and/or oncostatin M (*Rich* and *Leder*, 1995; *Clegg* et al., 1996; *Laky* et al., 1998). Many aspects of this extrathymic T cell developmental pathway are unresolved. It is for example unclear if lymphoid cells (and dendritic cells, DC) of the innate as well as the adaptive mucosal immune system originate at these sites.

Most specific, mucosal immune responses are induced in organised (structured and localised) structures of the small intestine gut-associated lymphoid tissue (GALT) called Peyer's patches (PP). Specific responses primed locally may mature locally in PP, or in regional mesenteric lymph nodes (mLN). Besides these major inductive compartments, innate or specific immune responses may be primed in the epithelial or lamina propria compartment of the mucosa. In these two diffuse compartments, effector cells, such as B- and T-lymphocytes, differentiated plasma cells, macrophages, and other antigen-presenting cells (APC's), as well as eosinophils, basophils, and especially mast cells are predominantly found.

The mucosal tissue represents an important interface between the innate and the adaptive immune system

A large array of different lymphoid cell subsets that are considered part of the innate or the specific (adaptive) immune system are found in the collections of effector cells of the diffuse GALT, i.e. the epithelium and the lamina propria (Table 1). Very little is known about the interaction of these subsets.

Lymphoid and myeloid cell populations in different compartments of the mucosal tissues are strikingly heterogeneous

Intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) of the gut are very heterogeneous in surface phenotype and function. To illustrate this point, Table 2 lists some of the subsets found in the murine small intestine IEL population. It is uncertain to what extent this heterogeneity reflects the presence of distinct lineages, distinct differentiation pathways, or distinct functional activation states that are unique to the mucosa. Peripheral $CD4^+$ $CD8^-$ TCR $\alpha\beta$ T cells that migrate from the intestinal lamina propria into the epithelial layer co-express $CD8\alpha$, and change their integrin and cytokine expression profile (*Reimann* and *Rudolphi*, 1995; *Morrissey* et al., 1995). This illustrates that peripheral T cells undergo distinct changes in phenotype as they traffic through different compartments of the mucosa.

Lymphoid and myeloid cell populations within different compartments of the mucosa display regional specialisation

The effector cells in diffuse compartments of the intestinal mucosa show striking regional specialisation. The small and the large intestine e.g. show a very different composition of effector cell populations in these compartments (*Camerini* et al., 1993; *Beagley* et al., 1995; *Boll* and *Reimann*, 1995a; *Boll* et al., 1995b).

GALT are subjected to tissue modelling (lymphoid neogenesis) dependent on cytokine networks and bacterial stimuli

GALT in germ-free animals are different from those in animals raised under conventional conditions. Data from genetically engineered mice indicate that ligands and receptors of the TNF superfamily are involved in forming the anlage and expanding rudimentary structures of the PP and the mLN. Especially lymphotoxin (LT) α and LTβ, the LTβ receptor (R) and other members of the TNFR receptor family play central roles in the differentiation of these tissues (*Koni* and *Flavell*, 1998). Although many details have not yet been resolved the available evidence indicates that cytokines play a decisive role in controlling the development of inductive sites of the mucosal immune system.

THE INNATE MUCOSAL IMMUNE SYSTEM

Innate immunity is often considered as a separate entity from the adaptive immune response. The current interest in innate immunity seeks to integrate these two distinct types of immune function (*Medzhitov* and *Janeway-CA*, 1997a; *Medzhitov*, *Janeway-CA*, 1997b). Examples for cells of the innate immune system that are found in mucosal tissues are listed in Table 1. The important point that emerges from this (incomplete) list is that the distinction between T cells belonging to either the innate, or the specific immune system is

Table 1: Cells of the innate and the adaptive immune system found in GALT

Innate immunity			Adaptive immunity		
Cells	Receptors	Ligand	Cells	Receptors	Ligand
CD8α cells	?	?	CD8αβT cells	αβ TCR	MHC-I/P
CD4 CD8αα T cells	αβ TCR	?	CD4 CD8αα T cells	αβ TCR	MHC-I,II/?
CD8αα T cells	γδ TCR	?hsp	CD4 T cells	αβ TCR	MHC-II/P
DN T cells	γδ TCR	?			
			NK1 CD4 T cells	αβ TCR	CD1/?
NK cells	?	?			
B1 cells	Ig	polyreactive	B2 cells	Ig	monoreactive
macrophages*	many	many			
mast cells*, eosinophils, basophils	many	many			
dendritic cells*	many	many			
Intestinal epithelial cells*	many	many			

P: peptide
hsp: heat shock protein
DN: double negative $CD4^-$ $CD8^-$
NK: natural killer
*: many different subsets

Table 2: Heterogeneity of T cell subsets within the murine small intestine IEL population

TCR[1]	CD3[2]	CD4	CD8[3]	Lineage[4]	Function[5]	Proportion[6]
αβ	ζζ	CD4+	CD8-	thymic	?	2-4%
αβ	ζζ	CD4+	CD8αα	thymic	tolerogenic	5-15%
αβ	ζζ	CD4-	CD8αβ	thymic	cytolytic	10-40%
αβ	ζ-FcεRγ	CD4-	CD8αα	extrathymic	epitheliotrophic	20-40%
γ δ	ζ-FcεRγ	CD4-	CD8αα	extrathymic	epitheliotrophic	20-60%
γ δ	ζ-FcεRγ	CD4-	CD8-	extrathymic	B help	5-10%

IEL: intraepithelial lymphocytes

1 the antigen receptor for T cells (TCR) is a heterodimer composed of eithe an α and β, or a γ and δ chain

2 the signal-transducing components of the CD3 complex can contain either the ζζ homodimer, or the ζ-FcεRγ

3 the CD8 coreceptor molecule can be expressed on the cell surface as either a CD8αα homodimer, or a CD8αβ heterodimer

4 T cells develop either in the thymus (thymic lineage), or at another (not well characterized) site (extrathymic)

5 IEL may support tolerance induction (tolerogenic), lyse cells (cytolytic), provide growth factors for epithelia (epitheliotrophic) or B cells (B help)

6 the proportion of IEL subsets listed are representative for the murine small intestine

not clear cut. MHC-II-restricted $CD4^+$ TCRαβ that co-express CD8 after homing to the epithelium, TCRγδ T cells (the recognition specificity of which is still unknown) or CD1-restricted $NK1^+$ $CD4^+$ TCRαβ T cells are examples of subsets that may be classified as cells of the innate or the specific immune system. This raises the question how this distinction has to be defined in the mucosal immune system. We will propose later that one way to view this dichotomy is to acknowledge that lymphoid cells of the immune system can be triggered by either innate, or specific stimuli. Hence the stimulus that drives the response, and not necessarily the distinct subset of lymphoid cells that respond, can classify a response as adaptive or innate. The signal for the adaptive (specific) and/or restricted response goes through the antigen receptor. Less is known about alternative ligand/receptor interactions that trigger innate immunity.

A large group of molecules of very different nature can activate T cells and antigen-presenting cells (APC) resulting in clonal expansion, differentiation and pro-inflammatory cytokine release (Table 3). These molecules are proteins, sugars, glycolipids, formylated peptides, oligodeoxy-nucleotides or lipopolysacchrides. Some of these ligands stimulate T cells similar to conventional MHC-restricted T cell recognition, e.g. the HM3 (MHC class Ib)-restricted presentation of formylated (f) peptides to TCRαβ T cells. The receptor systems and signal transduction cascades involved are often unknown but exciting new insight has been gained in the last years. These substances can be classified as adjuvants, as co-stimulators or as mitogens depending on the nature and state of differentiation/activation of the responding cell and the conditions (cofactors, concentration) of stimulation.

Table 3: Pattern recognition involved in the activation of the innate mucosal immune system

Ligand:	Butyric acid	hsp60	LPS	CpG ODNs	Mannan	?	Super antigen	Glycolipid	f-peptide
Cofactor:	?	?	LBP CD14	?	?	?	-	CD1	MHC-Ib (HM3)
Receptor:	NFκB	?	Toll-like R2 (TLR2)	?	DEC-205	costimulator (e.g. CD2)	TCR Vβ	TCRαβ	TCRαβ
Effect:	IL-1β	IL-6	TNFα IL-12	chemokines	T cell costimulation	T cell stimulation		T cell response	

Hsp: heat shock protein
LPS: lipopolysaccharide
f: formylated
LBP: lipopolysaccharide-binding protein
NF: nuclear factor

THE SPECIFIC MUCOSAL IMMUNE SYSTEM

Specific T cell-mediated mucosal immunity includes $CD4^+$ Th cell subsets, $CD8^+$ cytotoxic T lymphocytes (CTL), and specialised T-cell subsets for the induction of mucosal tolerance. Th cells control commitment of B cells to IgA production, their clonal expansion and their differentiation into plasma cells producing polymeric IgA (pIgA). T cells of the $CD4^+$ or $CD8^+$ phenotype are either naïve (have not yet encountered antigen), or activated (effector cells), or memory cells. The GALT contains a large reservoirs of precursor T cells for different subsets that allows the system to generate *in situ* potent $CD4^+$ Th cell and $CD8^+$ CTL responses after an encounter with bacterial or viral pathogens.

MODELS OF DYSREGULATED MUCOSAL $CD4^+$ T CELL RESPONSES LEADING TO CHRONIC INFLAMMATION

Aberrant immune responses can lead to mucosal inflammation, the chronic form of which is known as inflammatory bowel diseases (IBD). In man, IBD is manifest clinically as either ulcerative colitis (UC), or Crohn's disease (CD). Some animal models reproduce features of human IBD, and are thus of major interest to study the immunopathogenesis of this disease with the aim of developing novel therapeutic approaches. The models in mice and rats for the study of IBD that have emerged either through purposeful design of experimental protocols, or as unexpected 'by-products' of genetically engineered mouse lines, have been recently reviewed (*Conner* et al., 1994; *Bhan* et al., 1994; *Sartor*, 1994; *Reimann* et al., 1995a). Some of these models are listed in Table 4 where they are classified into three groups.

Models relying on local challenge with chemical irritants, immunostimulants or bacterial antigens

The local exposure of the colonic mucosa of mice and rats to the contact-sensitising agent trinitrobenzene sulphonic acid (TNBS) induces chronic colitis. Reactive TNBS modifies many proteins, a reaction that stimulates DTH responses to hapten (TNP)-modified self antigens. $CD4^+$ T cells producing the Th1 cytokines IL-2 and IFNγ stimulate the development of IBD in this model. Some inbred mouse strains exhibit greater susceptibility to disease induction, suggesting a genetic control in the development of IBD.

Genetic models.

Heritable models of colitis have been described in mice and monkeys. For example, the C3H/HeJBir substrain of C3H/HeJ mice develops a heritable (presumably multigenic) colitis early in life that spontaneously resolves with advancing age, and in which Th1 $CD4^+$ T cells apparently play a pathogenically relevant role. Chronic enteric inflammation is induced in transgenic or 'knock out' mice by manipulating T cells or cytokines by gene targeting. The most studied models include the chronic intestinal inflammation that develops in IL-2 knock-out mice, and the severe focal inflammation in both small and large intestines that is manifest in IL-10 knock-out mice. Both diseases are associated with an elevated local production of Th1 cytokines.

Adoptive transfer of immunocompetent $CD4^+$ T cells into syngeneic, immunodeficient hosts

Following the adoptive transfer of

Table 4: Animal models of inflammatory bowel disease (IBD)

I. administration of exogenous agents	
Chemical irritants:	acetic acid ethanol trinitrobenzene sulfonic acid (TNBS) oxazolone indomethacin s.c.
Immunostimulants	Freund's adjuvants immune complexes
Bacterial/synthetic antigens	peptidoglycan-polysaccharides (PG-PS) carrageenan dextran sulfate (DSS)
II. genetic models	
Spontaneous' disease	spontaneous ulcerative colitis in tamarin monkeys heritable murine colitis in C3H/HeJBir mice
Transgene expression	human HLA-B27/β2m transgene expression in SPF rats
Transgenic 'knock-out' models, deleted are:	
cytokine genes	IL-2, IL-10
TCR chains	TCR ß-chain, TCR δ-chain
signal transduction	Gαi2
adhesion molecule	E-cadherin
MHC class II	MHC class II α- and β-chain
multi-drug resistance transporter	mdr1a
III. adoptive $CD4^+$ T cell transfer into immunodeficient host (mice)	

$CD4^+$ T cells into severely combined immunodeficient (SCID) mice, the recipients develop a colitis that is characterised by a striking local expansion of Th1 $CD4^+$ T cells. This model has provided direct evidence that dysregulated Th1-type $CD4^+$ T cell responses are associated with murine IBD. We have studied this system, and have reviewed our data (*Reimann* et al., 1994; 1995a; *Reimann*, 1998).

INDUCTION OF T CELL-MEDIATED, CHRONIC MUCOSAL INFLAMMATION

Into young H-2^d C.B-17 *scid/scid* mice (SCID) mice, we transferred histocompatible, non-fractionated $CD4^+$ T cells from congenic C.B-17 +/+ or histocompatible BALB/c or BALB/c^{dm2} (dm2) mice. This reconstituted the hosts with gut-seeking $CD49d^{hi}$ $CD4^+$ T cells of the memory $CD44^{hi}$ $CD45RB^{lo}$ $CD62L^{lo}$ phenotype (*Rudolphi* et al., 1992; 1993a; 1996; *Reimann* et al., 1993; 1995b; *Rudolphi* and *Reimann*, 1993b; *Reimann* and *Rudolphi*, 1995; *Bonhagen* et al., 1996). A similar colitis was observed after heterotopic trans-

plantation of gut wall from an immunocompetent, histocompatible donor into the skin of the SCID host. The histopathology showed that $CD4^+$ T cell-repopulated SPF SCID mice developed inflammatory changes confined to the colonic mucosa. The small intestine down to the terminal ileum was usually devoid of inflammation.

$CD4^+$ T cells prepared from primary lymphoid organs (thymus), secondary lymphoid tissues (spleen; inguinal, popliteal or mLN) or peripheral tissues (lamina propria of the small or large intestine) displayed a comparable IBD-inducing potential. Gut lamina propria $CD4^+$ T cells from euthymic and athymic donor mice induced an IBD in the SCID host. Only $CD4^+$ T cells recovered from the gut epithelial layer of transplanted SCID mice showed a poor repopulation efficiency after transfer into secondary SCID hosts. Hence, following its transfer into the histocompatible SPF SCID host, all tested $CD4^+$ T cells repopulated the immunodeficient animal and induced an IBD (*Reimann* et al., 1995b; *Rudolphi* et al., 1996; *Claesson* et al., 1999).

$CD4^+$ T cells from normal rats expressing the $CD45RB^{high}$ phenotype have the potential to induce autoimmune diseases in congenic, immunodeficient hosts (*Fowell* et al., 1991; *Fowell*, Mason, 1993). Such $CD45RB^{hi}$ $CD4^+$ T cells from healthy animals thus seem to express an autoaggressive potential that can be revealed *in vivo*. $CD45RB^{hi}$ $CD4^+$ T cells from immunocompetent mice induced clinical and histopathological signs of colitis after transfer into a histocompatible, immunodeficient hosts (*Powrie* et al., 1993; 1994a; 1994b; 1996; *Morrissey* and *Charrier*, 1994; *Powrie*, 1995; *Leach* et al., 1996; *Aranda* et al., 1997; *Picarella* et al., 1997). We transferred polyclonal, oligoclonal or monoclonal $CD4^+$ TCR$\alpha\beta$ T cell populations into SPF SCID mice; the transfer of all three types of $CD4^+$ T cell populations induced an IBD in histocompatible SCID hosts (*Reimann* et al., 1995b; *Claesson* et al., 1999). The state of activation of the transferred $CD4^+$ T cells is relevant with respect of their IBD-inducing potential. Mitogen- or antigen-stimulated $CD4^+$ T lymphoblasts are more efficient in inducing IBD than the respective resting $CD4^+$ T lymphocytes (*Claesson* et al., 1999). Taken together, we have shown that many tested $CD4^+$ T cell subsets could induce an IBD after adoptive transfer into SCID hosts. There is thus little evidence that a particular $CD4^+$ T cell subset is involved but the relative efficiency with which different $CD4^+$ T cell subsets induced the disease varied.

IBD-ASSOCIATED $CD4^+$ T CELLS

Transferred $CD4^+$ T cells repopulate the lamina propria and epithelial layer of the small and large intestine, the mesenteric lymph nodes, the peritoneal cavity and the spleen (but not other peripheral lymph nodes or tissues) of the SCID host. Transfer of even high numbers of $CD4^+$ T lymphocytes or blasts into GF SCID mice did not repopulate the animals; a microbial gut flora is thus an essential prerequisite for successfully repopulating the SCID host with adoptively transferred $CD4^+$ T lymphocytes. The selective reconstitution of the immunodeficient host with gut-seeking $CD4^+$ T cells was confirmed by the surface phenotype of repopulating T cells: all T cells in the SCID host expressed the $\alpha_4\beta_7$ integrin (a homing receptor for mucosa-seeking leukocytes) (*Bonhagen* et al., 1998), and high levels of the P-selectin-binding ligand PSGL-1 (involved in leukocyte-endothelial cell interactions during intestinal inflamma-

tion) (*Thoma* et al., 1998). The repopulating, mucosa-seeking $CD3^+$ $CD4^+$ $CD8^-$ TCRαβ memory/effector cells express high levels of CD44, CD2 and CD28, but low levels of CD45RB and CD62L on the surface, and are $CD95^+$ (susceptible to apoptosis, or 'activation-induced cell death', AICD). A large fraction of these cells in the inflamed colonic lamina propria is *in situ* activated ($CD25^+$, $CD69^+$); the fraction of activated $CD4^+$ T cells increases strikingly with the progression of the disease. The gut lamina propria seems to be the major site of $CD4^+$ T cell proliferation (*Bregenholt* et al., 1998).

Transfers of limiting numbers of cells from polyclonal $CD4^+$ T cell populations into SPF SCID mice repopulate the host with T cell populations with a polyclonal TCRαβ repertoires; we detected no evidence for preferential expansion of oligoclonal populations in the immunodeficient, histocompatible host (*Rudolphi* et al., 1996). Even when 10^5 $CD4^+$ T cells (that were expanded in adoptive hosts for a 3-5 month period) were repeatedly 'passaged' through SCID mice, no reproducible bias in their TCR β-chain repertoire could be detected. We have not been able to select *in vivo* a $CD4^+$ T cell line with an enhanced IBD-inducing phenotype and a restricted TCR β-chain repertoire by repeated passage through different SCID hosts. Oligoclonal and monoclonal $CD4^+$ T cell lines were as efficient as polyclonal $CD4^+$ T cell lines in repopulating the SCID host and in inducing an IBD (*Claesson* et al., 1999). These data suggest that a TCR-independent (mitogen-like) stimulus drives T cell activation and expansion in the colonic lamina propria of transplanted SCID mice. The mitogenic stimulus that drives T cell expansion in the adoptive host is (directly or indirectly) dependent on the microbial flora of the gut.

Almost all $CD4^+$ T cells repopulating the adoptive SCID host produce the Th1 cytokines IFNγ and TNFα detectable either by ELISA (at the population level), or by intracellular staining and FCM analyses (at the single cell level). Very few (or no) IL4- or IL10-producing T cells are detectable. In the ELISA readouts, inducible IL12 levels in mononuclear cell population from repopulated tissues of the adoptive SCID host were high. Hence, the cytokine profile detectable in diseased, transplanted SCID mice displayed a pure Th1 phenotype, similar to that reported in other murine IBD models (*Powrie* et al., 1996; *Stuber* et al., 1996; *Berg* et al., 1996; *Neurath* et al., 1996a; 1996b; *Hörnqvist* et al., 1997; *Ehrhardt* et al., 1997; *Ludviksson* et al., 1997; *Strober* et al., 1997). Evidence has emerged for an IL10-producing immunoregulatory $CD4^+$ (Tr1) T cell subset that prevents Th1 differentiation and may be an important physiological control for preferential Th2 type differentiation in mucosal immune responses (*Groux* et al., 1997). The observation that IL-10 is absent from transplanted SCID mice supports this concept.

The $CD4^+$ T cell-mediated immune effector mechanisms that damage the mucosal tissue are unknown. Th1 $CD4^+$ T cells isolated from the colonic lamina propria of transplanted SCID mice are cytolytic (*Bonhagen* et al., 1996; *Boirivant* et al., 1996; *De* et al., 1996). The TCR-mediated cytolytic effect operates through the CD95(Fas)/CD95L(FasL) pathway. The CD95-expressing targets of this cytolytic attack that are relevant in the pathogenesis of IBD are intestinal epithelial cells (IEC), APC and the $CD4^+$ T cells themselves. A central role of epithelial damage in the pathogenesis of IBD as a result of the immune attack by T cells has been postulated (*Iwamoto* et al., 1996; *Sträter* et al., 1997; *Sakai* et al., 1997). Some $CD95^+$ APC are susceptible to $CD4^+$ T cell attack which may select for APC in the colonic lamina propria that bias the response towards

Table 5: Colonic lamina propria T cell activation, expansion and apoptosis *in vivo* in transplanted SCID mice with IBD

T cell activation and expansion
• an increasing fraction of $CD4^+$ T cells is activated *in situ* ($CD69^+$ $CD25^+$) in the colonic lamina propria during progression of the IBD • $CD4^+$ T cells rapidly proliferate mainly in the colonic lamina propria • transferred $CD4^+$ T cells expand >100-fold in number in the adoptive host • freshly explanted colonic lamina propria $CD4^+$ T cells show cytokine-dependent (IL-2/IL-7) proliferation *in vitro*
T cell apoptosis and cytolytic reactivity
• colonic $CD4^+$ T cells are exquisitely susceptible to 'activation-induced cell death' (AICD) *in vivo* and *in vitro* • AICD is triggered *in vitro* by TCR/CD3 ligation (? are $CD4^+$ T cells selected *in vivo* against gut flora-specific reactivities) • CD95/CD95L (Fas/FasL) is involved in AICD of colonic lamina propria $CD4^+$ T cells in IBD
specific *versus* non-specific T cell activation
• the TCR-Vß repertoire of colonic $CD4^+$ T cell populations from diseased SCID mice suggests that they are polyclonal even after repeated transfers through SCID hosts • *in vivo* selection of oligoclonal $CD4^+$ T cell lines with a restricted TCR-Vß repertoire and an enhanced IBD-inducing potential by repeated passages of limiting numbers of $CD4^+$ T cells through SCID hosts was repeatedly unsuccessful • adoptive transfers of oligoclonal $CD4^+$ T cell lines induce colitis

the Th1 direction. We detected massive AICD in 50-70% of the colonic lamina propria $CD4^+$ T cells from transplanted SCID mice *in situ*, in gut-derived $CD4^+$ T cells explanted *in vitro*, and in gut-derived $CD4^+$ T cell populations after *in vitro* TCR-dependent stimulation (*Bonhagen* et al., 1996; 1998; *Bregenholt* et al., 1998). In addition, pro-inflammatory mediators seem to damage stroma cells and the integrity of the mucosal barrier, and to destroy the tissue matrix.

INTERPRETATION OF DATA FROM THE MODEL

The available data represent indirect evidence that a TCR-independent, mitogenic stimulus drives the polyclonal activation, expansion and differentiation of colonic lamina propria $CD4^+$ T cells in the diseased, adoptive host. Because $CD4^+$ T cell repopulation and expansion is only observed in mice with an intact gut flora, a microbial factor has to be a key player that (directly or indirectly) triggers, amplifies and/or directs the T cell response in the colon.

Chronic development of the colitis is associated with a massive infiltration of the colonic lamina propria with donor-type $CD4^+$ T cells. An increasing fraction of these T cells shows evidence of *in situ* activation (i.e. expression of CD69 and CD25) as the disease progesses. We have demonstrated that the transferred $CD4^+$ T cells expand >100-fold in number in the adoptive host. *In vivo* labelling indicates that the colonic lamina propria is the major site of T cell

proliferation in the transplanted SCID mouse. Freshly explanted colonic lamina propria $CD4^+$ T cells from transplanted SCID mice with IBD proliferated *in vitro* in the presence of the cytokines IL-2 and IL-7 (that both are abundantly present in the colonic lamina propria).

Activation and proliferation of colonic $CD4^+$ T cells is accompanied by extensive *in situ* cell death by apoptosis. Histochemical evidence (TUNEL staining) indicates that 50-80% of the lymphoid cells infiltrating colonic lesions are undergoing apoptosis. Lamina propria macrophages contain abundant numbers of tangible bodies indicating their phagocytosis of apoptotic lymphoid cells. All colonic $CD4^+$ T cells are $CD95^+$, and a fraction of 20-30% of these cells express the CD95L. We presented evidence that colonic $CD4^+$ T cells are exquisitely susceptible to 'activation-induced cell death' (AICD) *in vitro*, and that CD95/CD95L (Fas/FasL) interactions are involved in AICD of these T cells in IBD. This AICD is triggered *in vitro* by TCR/CD3 ligation. This makes it unlikely that restricted, antigen-specific stimulation of T cells via the TCR drives this massive T cell expansion. The inverse scenario seems more compatible with the data: a mitogenic stimulus drives the cytokine-dependent, polyclonal proliferation of these $CD4^+$ T cells, and the TCR-dependent, specific and restricted stimulation of the activated T cells by antigens from the gut flora triggers specific deletion of T cells. In this way, the repertoire of the activated and proliferating $CD4^+$ T cell population would be selected *in vivo* against gut flora-specific reactivities.

The data on the TCR-Vβ repertoire of colonic $CD4^+$ T cell populations from diseased SCID mice (Table 5) are compatible with this interpretation. IBD-associated colonic lamina propria $CD4^+$ T cell populations are polyclonal. They remain polyclonal even after repeated 3-4 month transfers through SCID hosts in which they repeatedly induced the disease. Although such $CD4^+$ T cell lines that were passaged *in vivo* for 22 months through 6 generations of SCID hosts, went repeatedly through population 'bottlenecks' in the course of these transfers (only 10^5 $CD4^+$ T cells were injected to initiate each new transfer), this *in vivo* selection did not result in the appearance of a reproducible bias in the TCR-Vβ repertoire in the $CD4^+$ T cell populations. We have furthermore demonstrated that adoptive transfers of oligoclonal $CD4^+$ T cell lines of different origin (i.e. from different types of transgenic donor mice) are equally efficient in inducing a colitis. Taken together, these data suggest that a mitogenic (gut flora-dependent) stimulus drives the polyclonal activation and expansion of TCRαβ $CD4^+$ T cells in the colonic lamina propria while the specific and restricted recognition tends to delete the respective T cell clone from the GALT.

THE INNATE/SPECIFIC IMMUNITY INTERFACE

Are there precedents for the TCR/CD3-independent activation of LPL? Evidence is emerging that this is in fact the case. Some ligands bind to surface receptors of lymphocytes and thereby trigger activation. The CD28 molecule expressed by T cells is considered a co-stimulator molecule, i.e. coligation of this determinant with specific antigen recognition triggers T cell activation. Different groups have reported that monoclonal antibodies specific for CD28 trigger T cell proliferation and cytokine gene expression *in vitro* and *in*

Table 6: Alternative (TCR-independent) activation of T cells of the specific (adative) immune system

T cell receptor	ligand
CD2	CD58 (LFA-3), CD48
CD28	CD80 (B7-1), CD86 (B7-2)
integrin $\alpha E\beta 7$	E-cadherin
? surface receptor	Hsp70, hsp96
? intracellular target	butyric acid
? intracellular target	ISS CpG-containing ODN

Hsp: heat shock protein
ISS: immune stimulating sequences
ODN: oligodeoxynucleotides

vivo (*Tacke* et al., 1997; *Flynn* and *Mullbacher*, 1997; *Siefken* et al., 1997; 1998). These data suggest that binding of CD80 or CD86 (expressed by APC) to CD28 (expressed by T cells) can induce T cell proliferation without TCR/CD3 involvement. Interaction of the integrin $\alpha E\beta 7$ on the surface of IEL with E-cadherin on the surface of IEC can activate the former in an antigen receptor-independent manner (*Sarnacki* et al., 1992). The TCR-independent activation *in vivo* and *in vitro* of T cells by antigen-negative heat shock proteins (hsp) of the 70 and 96 kDa class has been demonstrated (*Breloer* et al., 1999). Activation of the CD2 pathway in lamina propria T cells up-regulates IL-2 expression in human LPL (*Gonsky* et al., 1998). Microbial products may mimic some of these ligand/receptor interactions and may thereby activate T cells. Other reagents may readily cross the cell membrane and interfere with signal transduction cascades. E.g. butyric acid listed in Table 3 is known to activate NF-κB. There may thus be a plethora of antigen receptor-independent activation pathways for lymphocytes of the specific immune system, in particular for T cells.

Effector functions (e.g. release of cytokines or cell contact-dependent cellular interactions) do not differ between the innate and the specific immune system. Their difference is generally assumed to be based on the way the system is triggered: specific *versus* non-specific activation. We presented evidence that the specific T cell system seems to be activated non-specifically on an impressively large scale in the mucous membranes of the gut. We may therefore have to modify our view of these systems. Instead of conceiving them as separate, interacting systems, we may have to think of them as the alternative activation pathways of an old (innate) and a new (specific) way to stimulate lymphocytes. The choice for the activation pathway operating may depend on the particular micro-environment it resides in. The mucosal immune system as the primary defence site to foreign challenges may imprint a predominance of innate reactivity. Its strength may be to focus all defences to the quick, limited, short lived responses. This may limit the importance of specific immune responses in the intestinal mucosa.

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INTRAVENOUS IMMUNOGLOBULIN (IVIg) IN AUTOIMMUNE DISEASES – EXPANDING INDICATIONS AND INCREASING SPECIFICITY

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SUMMARY

Even though intravenous immunoglobulin (IVIg) was originally used for the correction of immunodeficiency states, in the recent years it is also used as an immunomodulating agent in several autoimmune diseases. The indications for IVIg use in autoimmunity progressively expand and might include for example systemic lupus erythematosus, autoimmune vasculitides, and antiphospholipid syndrome. However, as disease indications expand, IVIg should be used in specific situations that depend on both patients' variables (e.g. clinical manifestations) and on IVIg-related variables (e.g. concentration of specific anti-idiotypes).

INTRODUCTION

IVIg are composed of immunoglobulins, mainly of the IgG isotype, produced from numerous donors. Whereas its first indication was various immunodeficiency states, it is currently an accepted treatment also for immune thrombocytopenic purpura, Kawasaki disease, Guillain-Barré syndrome, and polymyositis/dermatomyositis.

IVIg IN AUTOIMMUNE DISEASES – EXPANDING INDICATIONS

In addition to the above-mentioned diseases, there are several reports of IVIg use in other autoimmune diseases. The persistent reports of clinical success in the treatment of autoimmune diseases with IVIg, combined with the understanding of the mechanisms of action of IVIg, result in expansion of the possible indications for its use as an immunomodulating agent (Figure 1).

Literature review

Herein we concentrate on the literature reports of IVIg in vasculitis and systemic lupus erythematosus (SLE). The patients included in the case-series of IVIg in vasculitis had Wegener's granulomatosis, microscopic polyangiitis, rheumatoid vasculitis, parvovirus B19-associated polyarteritis nodosa, IgA nephropathy, and Henoch-Schönlein purpura. The largest case series of IVIg therapy in vasculitis included 26 patients: 14 with Wegener's granulomatosis, 11 with microscopic poly-angiitis, and a patient with rheumatoid vasculitis (*Jayne* and *Lockwood*, 1993). The response rate to IVIg reported in this serious was 100%, as in smaller case-series of 3-11 patients

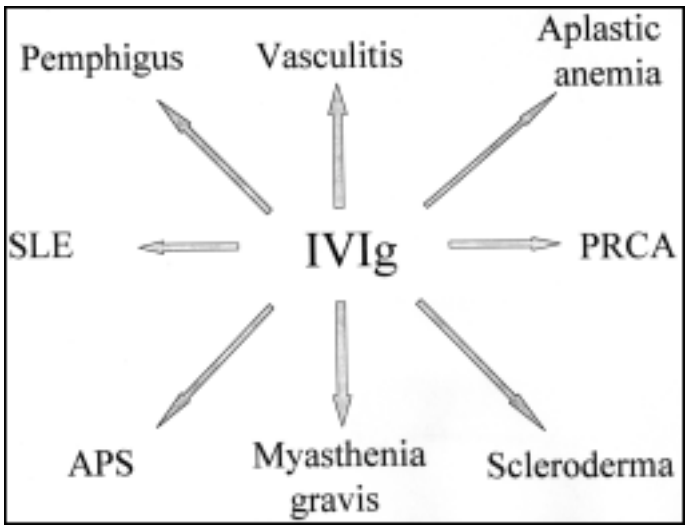

Figure 1: Expanding indications for IVIg use in autoimmunity.
The indications for IVIg use in autoimmunity progressively expand. They currently include immune thrombocytopenic purpura, Kawasaki disease, polymyositis/dermatomyositis, and Guillain-Barré syndrome. IVIg may be a therapeutic option in several other autoimmune conditions.
APS = Antiphospholipid syndrome
PRCA = Pure red cell aplasia
SLE = Systemic lupus erythematosus

(*Finkel* et al., 1994; *Jayne* et al., 1991; *Jayne* and *Lockwood*, 1996; *Rostoker* et al., 1994). However, in other series of 9-15 patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated systemic vasculitides, the response rate was only 40-55% (*Richter* et al., 1993; 1995).

The various clinical manifestations of SLE that were reported to be successfully treated by IVIg in case-reports include: autoimmune haemolytic anaemia (*Marmont*, 1983; *Roldan* et al., 1994), thrombocytopenia (*Roldan* et al., 1994; *Ruiz-Valverde* et al., 1994; *Lseprit* et al., 1996), pancytopenia (*Akashi* et al., 1990), pleural effusion (*Ben-Chetrit* et al., 1991), pericarditis (*Hjortkjoer Petersen* et al., 1990), nephritis (*Akashi* et al., 1990; *Oliet* et al., 1992; *Winder* et al., 1993; *Welch* et al., 1995), pure red cell aplasia (*Ilan* and *Naparstek*, 1993), secondary antiphospholipid syndrome with cerebral infarction (*Strufelt* et al., 1990), end-stage renal disease (*Becker* et al., 1995), pneumonitis and encephalitis (*Winder* et al., 1993), polyradiculoneuropathy (*Lesprit* et al., 1996), acquired factor VIII inhibitors (*Lafferty* et al., 1997), and cardiogenic shock (*Disla* et al., 1993). There are also few case series of IVIg use in SLE patients (*Corvetta* et al., 1989; *De Pita* et al., 1997; *Francioni* et al., 1994; *Lin* et al., 1989; *Maier* et al., 1990; *Schroeder* et al., 1996) in which the response rate to IVIg therapy ranged from 33% (3 of 9) to 100% (5 of 5), while the 2 largest series included 12 patients each.

Personal experience

We have recently concluded a clinical study in which we treated patients with various autoimmune diseases with IVIg. These diseases included: SLE, vasculitis, immune thrombocytopenic purpura, Guillain-Barré syndrome, polymyositis/dermatomyositis, haematologica

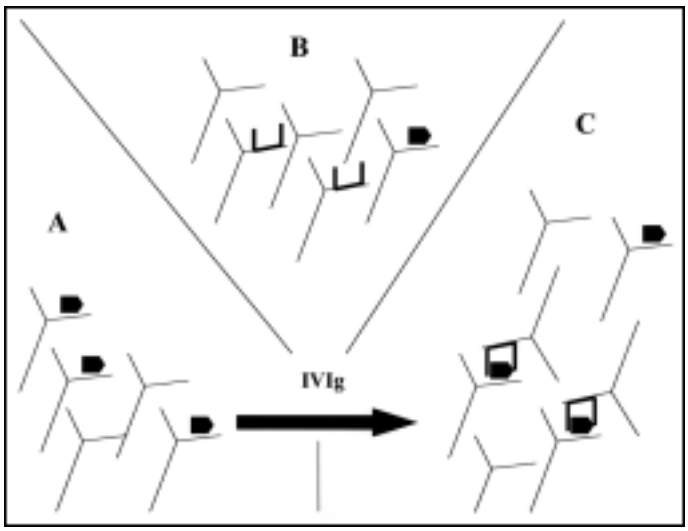

Figure 2: Anti-idiotypic activity of IVIg.
1st- The plasma of patients with autoimmune diseases (e.g. SLE) contains pathogenic idiotypes that are either absent in healthy subjects or are found in decreased concentrations.
2nd- IVIg, a plasma pool from numerous donors, contains many idiotypes and anti-idiotypes.
3rd- Anti-idiotypes within IVIg preparations bind pathogenic idiotypes in the patient's plasma and neutralise them, concomitant with clinical improvement. IVIg restores the normal balance between idiotypes and anti-idiotypes found in normal subjects.

autoimmune diseases (e.g. autoimmune haemolytic anaemia, pure red cell aplasia, aplastic anaemia, Evan's syndrome), and pemphigus vulgaris. We have treated 10 patients having vasculitis (2 with Wegener's granulomatosis, 2 with Churg-Strauss vasculitis, 2 with livedo vasculitis, and 4 with other systemic vasculitides) with 1-6 courses of IVIg to which 6 patients had a beneficial response (*Levy* et al., 1999). The clinical manifestations of several SLE patients treated by us with IVIg are myelofibrosis (*Aharon* et al., 1997), psychosis (*Tomer* et al., 1992), a patient with cytopenia, nephritis and serositis (*Aharon* et al., 1994), pleural effusion (*Sherer* et al., 1999a), myocardial dysfunction (*Sherer* et al., 1999b), and neuropsychiatric lupus (*Sherer* et al., 1999c).

IVIg IN AUTOIMMUNE DISEASES – INCREASING SPECIFICITY

The impressive clinical reports of beneficial response to IVIg in various autoimmune diseases strongly suggest that IVIg might be the treatment of choice in some of these cases. However, as the disease-indications for IVIg use would expand, it might be beneficial in these diseases in specific situations rather than in every case. Hence, future clinical research of IVIg should concentrate on identifying the patients subpopulations that would benefit most from IVIg, based on clinical presentation, laboratory parameters, response to other therapeutic modalities, or a combination of these. Examples for these variables include our preliminary data of IVIg in SLE that discloses that 8 of 9 patients with fever and arthritis had a beneficial response to IVIg. In addition,

the various laboratory parameters measured in the case-series of IVIg in SLE (*Corvetta* et al., 1989; *De Pita* et al., 1997; *Francioni* et al., 1994; *Lin* et al., 1989; *Maier* et al., 1990; *Schroeder* et al., 1996) emphasise that in general, IVIg therapy led to decreased anti-ds-DNA antibody levels, increased C3, C4 and total complement haemolytic activity, and no change in antinuclear antibody level, antibodies to RNP, Sm, and SSA/SSB. Finally, it has been claimed that patients with immune thrombocytopenic purpura who have good or excellent responses to IVIg are likely to similarly respond to splenectomy, as the spleen is a major site of macrophages with Fc receptors and platelet destruction (*Law* et al., 1997). This exemplifies the need for larger patient-series in order to determine who are the best candidates to IVIg therapy.

Patient selection for IVIg therapy is however only one aspect of increased specificity in IVIg therapy. Apart from the patient's characteristics, the specificity can be increased by modification of IVIg preparations. Examples for the mechanisms of action of IVIg include regulation of the idiotypic network, enhanced suppressor activity, Fc receptor blockade, complement regulation, and T-cell regulation (*Ballow*, 1996). Idiotypic network modulation is involved both in the pathogenesis and the treatment of various autoimmune diseases. There is a strong evidence for the presence of anti-idiotypes in IVIg preparations. For example, IVIg was shown to have anti-idiotypic activity both to anti-DNA and anti-cardiolipin antibodies, and in an animal model IVIg infusion succeeded in decreasing and ameliorating of experimental SLE and antiphospholipid syndrome, and resulted in a decrease of the respective antibodies to within normal levels (*Bakimer* et al., 1993; *Krause* et al., 1995). As emphasised in Figure 2, the anti-idiotypes within IVIg preparations bind pathogenic idiotypes in the patient's plasma (which is deficient in anti-idiotypes) and reverse the imbalance between idiotypes and anti-idiotypes which is followed by clinical improvement. Since the vast majority of the IVIg preparations contain polyclonal IgG rather than anti-idiotypes to pathogenic autoantibodies, it is natural to speculate that use of isolated anti-idiotypes will result in a superior effect than that of IVIg. Therefore, the specificity of IVIg therapy might also be increased by the formation of super-IVIg: IVIg preparation that contains increased concentration of anti-idiotypes to pathogenic idiotypes. Moreover, as different patients having the same disease can have different pathogenic idiotypes, as occurs in SLE, future immunotherapy with IVIg might be patient-specific with preparation of a cocktail of IVIg enriched with anti-idiotypes to the very same idiotypes found in every patient's plasma.

CONCLUSION

Nowadays IVIg is an immunomodulating agent used for the treatment of a few autoimmune diseases. However, the indications for IVIg use progressively expand. Since patients respond differently to IVIg, and currently there is no reliable way to predict who would benefit from this treatment, an effort should be carried out in order to predict who might enjoy from IVIg. The means of increased specificity of IVIg therapy should include both patient-related variables (clinical manifestations, laboratory parameters, response to other therapeutic modalities) and drug-related characteristics such as specific idiotypes' concentration within IVIg.

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INTRAVENOUS IMMUNOGLOBULIN (IVIg) AS AN INHIBITOR OF TUMOUR GROWTH: FROM AUTOIMMUNITY TO CANCER

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SUMMARY

The relationship between autoimmunity and cancer is described in this manuscript from the pathogenic and therapeutic point of view. In our view, autoantibodies derived from patients with autoimmune conditions, may be utilised for cancer treatment. In addition to these therapeutic modalities, IVIg is an example of how the autoimmune-cancer relationship generated novel treatment for cancer. The efficacy of IVIg as an inhibitor of tumour spread was shown in experimental murine models of melanoma, sarcoma and carcinoma. The mechanism through which IVIg acts entail its capability to induce IL-12 production (an anti-cancer and anti-angiogenic factor) and a subsequent activation of natural killer cells (NK), the presence of antibodies against tumour associated antigens within the IVIg preparations as well as antibodies against adhesion molecules. It may thus be concluded that IVIg exerts its anti-cancer activity through multifactorial mechanisms involving each step in the process of metastatic spread.

INTRODUCTION

Cancer and autoimmunity share similar aetiological and pathological mechanisms such as uncontrolled cell proliferation, impaired apoptotic pathways, cytokine disregulation, hormonal balance alterations, changes in membrane adhesion molecule expression etc. Autoimmune conditions and malignancy co-exist frequently: Cancer may develop in patients with autoimmune diseases, while autoimmune conditions may follow malignancy (*Sela* and Shoenfeld, 1988; *Swissa* et al., 1990).

Haematological malignancies, including leukaemia, Iymphoma, Hodgkin's disease and multiple myeloma may follow autoimmune diseases (*McCarty*, 1985; *Vainio* et al, 1983; *Shoenfeld* et al., 1983). The strongest association is found between Sjögren's syndrome and lymphoma. The lymphomas are usually of the B cell origin, although T cell lymphomas have also been found (*Mouydopoulodan*, 1992; *Chevalier* et al., 1991). Patients with rheumatoid arthritis (RA) have a 2-3 times greater risk of developing lymphoproliferative malignancy, even in the absence of immunosuppressive therapy. The risk is further increased following treatment with cytotoxic drugs (*Pries*, 1985; *Prior*, 1985). Similarly, systemic lupus erythematosus (SLE) has been associated with lymphoma both in animal models, such as NZB and MRL/lpr, and in humans (*Green* et al., 1978; *Wyburn-*

Table 1: Neoplasms in autoimmune conditions

Malignancy	Autoimmune disease
Lymphoproliferative malignancy	Sjögren's syndrome, SLE, RA
Thymoma	myasthenia gravis
Lung cancer	scleroderma, PM, DM
Breast carcinoma	scleroderma, stiff-man syndrome, DM, PM
Gynaecologic carcinoma	DM, PM

Mason, 1979; *Berliner* et al., 1983).

Other autoimmune conditions may be associated with different malignancies. The most prominent examples are myasthenia gravis and high incidence of thymoma (*Wu* and *Low*, 1996) systemic sclerosis (scleroderma) with lung cancer or with breast carcinoma (*Davis* et al., 1996; *Winkelmann* et al, 1988) and stiff-man syndrome in breast cancer (*Rosin* et al., 1998). Three additional autoimmune diseases - mixed connective tissue disease (MCTD), polymyositis (PM) and dermatomyositis (DM) - may be associated both with haematological neoplasms and with epithelial tumours (*Black* et al., 1982; *Schulman* et al., 1991; *Seda* and *Alarcon*, 1995; *Maoz* et al., 1998). Table 1 summarises the different malignancies and the associated autoimmune conditions.

Several explanations were introduced for the induction of malignancy in autoimmune conditions: susceptibility of the patients to both diseases (*Shoenfeld* and *Shwartz*, 1984; *Schreinemachers* and *Everson*, 1994); immunological predisposition (*Raubinain* and *Talal*, 1978; *Mountz* et al., 1984); oncogene activation and expression (*Kinlen*, 1985); the treatment of autoimmune diseases with immunosuppressive drugs may induce Iymphoproliferation and even trigger other tumour growth (*Fishman*, 1994).

Another association between autoimmunity and cancer is the occurrence of autoantibodies in patients with both haematological and epithelial malignancies. *Swissa* et al. (1990) examined different autoantibodies in the sera of 150 lymphoma patients and 164 cancer patients. Antinuclear antibodies (ANA) were detected in leukaemia. Anti-ss-DNA, anti-RNP and anti-Sm were found in the sera of patients with lymphoma. Among patients with epithelial malignancies, those with breast cancer had ANA and anti-smooth muscle antibodies. Lung cancer patients had anti-smooth muscle antibodies, antineuronal antibodies and autoantibodies to fibrillar collagen (*Lucchinetti* et al., 1998; *Fernandez* et al, 1996). Patients with head and neck carcinoma having higher serum immunoglobulin IgA levels, also exhibit IgA-anti-F(ab')2 autoantibodies (*Lorenz* et al., 1988) and patients with hepatocellular carcinoma have antinuclear antibodies (*Covini* et al., 1977).

Our group have recently defined tyrosinase, an enzyme which participates in the process of melanin production, as an autoantigen in vitiligo (*Baharav* et al., 1996). Autoantibodies to tyrosinase were detected in melanoma with a correlation to disease stage and to the development of white patches on the patient's skin (*Fishman* et al., 1997). Table 2 summarises the various autoantibodies which were defined in cancerous diseases.

It was suggested that the generation of autoantibodies in malignant condi-

Table 2: Autoantibodies in malignancy

Malignancy	Autoantibodies
Lymphoma	anti-ssDNA, anti-RNP, anti-SM
Breast carcinoma	ANA, anti-smooth muscle
Lung cancer	anti-smooth muscle, anti-neuronal, anti-fibrillar, collagen
Head & neck carcinoma	IgA-anti-F(ab')2
Hepatocellular carcinoma	ANA
Melanoma	tyrosinase

tions are an aspect of immune deficiency or an immune response against proteins which are involved in proliferative functions.

The common treatments aiming at similar targets and therapies based on the understanding of immune mechanisms in both diseases, are the subject of the current article.

An improved understanding of immune mediated tumour suppression should therefore greatly benefit immunotherapy of autoimmune diseases, and the two areas of research would benefit from an interdisciplinary endeavour.

COMMON THERAPIES FOR CANCER AND AUTOIMMUNITY

Similar strategies based on disease pathogenesis are employed to combat autoimmunity and cancer. The main pathways to be attacked in autoimmunity are modulation of immunoglobulin production, immunosuppression and interfering with inflammatory reactions. In cancer, tumour cell proliferation inhibition, immune system stimulation and metastasis prevention are targeted.

Shared pathogenic mechanisms lead to the implementation of therapies used in cancer for the treatment of autoimmune diseases and vice versa. The followings are some common treatments for both diseases.

Chemotherapy

Uncontrolled cell proliferation is shown in both cancer and autoimmunity. Chemotherapy, the gold standard for the therapy of malignant conditions, is used in cancer to inhibit tumour cell growth and in autoimmune conditions to modulate proliferating B cells producing pathogenic antibodies. Two cytotoxic agents, cyclophosphamide and methotrexate act via inhibition of nucleotide biosynthesis and are widely used in cancer therapy and in some autoimmune conditions. The clinical use of cyclophosphamide in autoimmunity is for the treatment of renal and cerebral lupus, vasculitis, especially in the context of Wegener's granulomatosis and rheumatoid arthritis. Methotrexate is used for the treatment of rheumatoid arthritis and several malignant conditions. It inhibits the enzyme dihydrofolate reductase. It exerts an anti-inflammatory effect through its capability to induce adenosine release in sites of inflammation (*Cronstein* et al., 1995; *Bouma* et al., 1994). Adenosine has an anti inflammatory effect due to its capability to inhibit the production of inflammatory cytokines such as TNF-α, IL-6, and IL-8 (*Tey* et al., 1992). Extracellular adenosine was shown by us (*Fishman* et al., 1998) and others (*Tey*

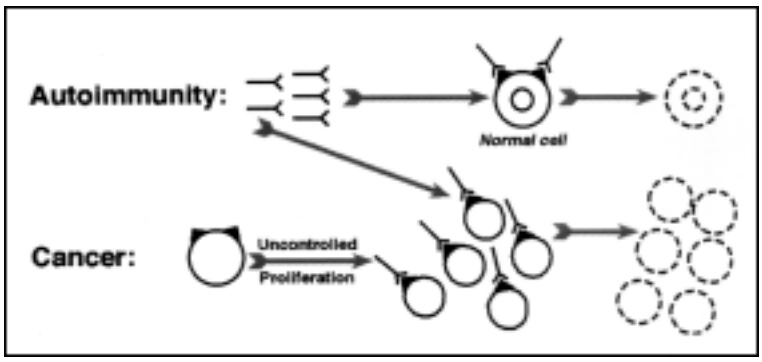

Figure 1: Autoantibodies from patients with autoimmune diseases bind and destroy normal cells presenting certain autoantigens. Such autoantibodies will bind to, and destroy the respective cancer cells which are of the same cellular origin as the normal cells and display the same autoantigens.

et al., 1992) to act as an anti cancer agent by binding to specific cell surface receptors and to inhibit specifically tumour cell proliferation. Thus, adenosine which acts as both an anti-inflammatory agent and an inhibitor of tumour cell growth, may serve as a treatment for cancer as well as autoimmune conditions.

Non-steroid anti-inflammatory drugs (NSAIDs)

Aspirin and other NSAIDs are widely used for the treatment of autoimmune conditions and are considered as potent anti-inflammatory drugs. These agents act by blocking the enzymes cyclo-oxygenase I and II, thus inhibiting the synthesis of prostaglandins and Thromboxane A2 (TXA2). Recently, we reported a novel mechanism by which a low dose aspirin was found to be extremely beneficial for the treatment of anti-phospholipid syndrome (APS). This syndrome is characterised by repeated thrombo-embolic phenomena and recurrent foetal loss. Our studies indicated that only low dose aspirin affected and prevented the pregnancy loss by increasing production of interleukin-3. We demonstrated that this cytokine is deficient in mice with experimental APS and has instrumental role in normal pregnancy (*Fishman* et al., 1992; *Fishman* and *Shoenfeld*, 1993).

Individuals who regularly take aspirin or other NSAIDs have been reported to be at reduced risk for the development of cancers of the colon (*Thun* et al., 1991; *Thun* et al., 1993; *Giovannucci* et al., 1995; *Berkel* et al., 1996) and possibly other sites including the stomach, oesophagus, lung and breast cancer (*Farrow* et al., 1998; *Schreinemachers* and *Everson*, 1994). NSAIDs exert their anti-cancer activity through the inhibition of prostaglandin synthesis, blockade of prostaglandin induced immunosuppression (thereby enhancing immune response) and induction of apoptosis and inhibition of tumour cell proliferation (*Samaha* et al., 1997; *Elder* et al., 1996). Aspirin was also found to stimulate the production of interleukin-12, a potent cytokine with anti-cancer activity, known to activate NK and cytotoxic lymphocytes which subsequently combat tumour cells (unpublished data).

Bone marrow transplantation

Bone marrow stem cell transplantation is currently used to treat patients with haematological and other malignancies such as breast and ovarian neoplasms. Autografts or allografts are

transplanted to cancer patients allowing the administration of high dose chemotherapy or radiotherapy and confer an anti-tumour effect separate of the chemotherapy effects. With the clinical development of haematopoietic growth factors, peripheral blood derived stem cells can be transplanted instead of bone marrow stem cells (*Blank* et al., 1995). Autoimmune conditions seems to be a stem cell disease and therefore can be transferred by bone marrow transplantation (*Blank* et al., 1995; *Sherer* and *Shoenfeld*, 1998). Yet it seems currently that if there is any cure for autoimmune conditions it is following heterologous bone marrow transplantation after total B and T cell ablation or autologous transplantation after proper purging of pathogenic T cells. Indeed the experience with patients seems encouraging although as yet, has not been widely tested.

Idiotypes and anti-idiotypes

Idiotypic immunomodulation is an additional harnessed therapy in both cancer and autoimmunity. The idiotypic network is an important mechanism for controlling the immune system (*Shoenfeld* et al., 1997; *Jerne*, 1974) and autoimmune diseases may be attributed to the disturbances of the network. Thus, one may speculate that manipulation of idiotypes ("pathogenic", "cross-reactive") of autoantibodies (anti-idiotypic immunity), may be effective in the treatment of autoimmune diseases.

Indeed, there are encouraging reports which show the beneficial effect of anti-idiotypic antibodies in the treatment of B cell tumours (*Levy* and *Miller*, 1990). In this approach, the target of the anti-idiotypic antibodies is the tumour specific antigen which is the idiotype of the cell surface immunoglobulin present on B cells. This modality was expanded to the use of anti-idiotypes generated against tumour associated antigens (*Merimsky* et al., 1997; *Blank* et al., 1994). Anti-idiotypes can directly regulate autoantibodies or indirectly, following active immunisation with a dominant idiotype. Needless to say, IVIg (see below), is also considered to affect autoimmune diseases by its content of polyspecific anti-idiotypes.

HARNESSING AUTOREACTIVITY FOR CANCER TREATMENT

In this section two therapeutic approaches making use of the "positive" relationship between autoimmunity and cancer, are discussed.

The first presents the use of autoantibodies derived from patients with autoimmune diseases as a potential therapy for cancer and the second introduces intravenous gamma globulin (IVIg), a common therapy for autoimmune diseases, as a treatment for the prevention of tumour metastases.

Autoantibodies

Autoantibodies in patients with autoimmune diseases are capable of binding and destroying normal cells presenting certain autoantigens. We presented recently a concept (*Fishman* et al., 1997) based on the realisation that such autoantibodies will bind to and destroy the respective cancer cells which are of the same cellular origin as the normal cells and display the same autoantigens (Figure 1).

This concept is wide and applicable for many autoimmune diseases and diverse malignant conditions. To illustrate the therapeutic and practical potential of this novel linkage several examples of autoantibodies and the respective cancer cells are depicted in Table 3 and are detailed below.

Table 3: Pairs of autoimmune diseases and the respective neoplasms

Autoimmune disease	Cancer disease
Vitiligo anti-melanocyte Abs.	Melanoma
Autoimmune haemolytic anaemia anti-red blood cell Abs.	Polycythaemia vera, Erythro-leukaemia
Antiphospholipid syndrome anti-phosphatydilserine Abs.	Cancer cells exhibiting phosphatydilserine on the outer cell membrane
SLE , other auto immune diseases anti-lymphocyte Abs.	Chronic lymphocytic leukaemia
Pemphigus vulgaris anti-keratinocyte Abs.	Squamous cell carcinoma of the skin

Vitiligo and melanoma

Vitiligo is a dermatologic autoimmune disorder presented as depigmented skin areas. The destruction of the pigmented cells (melanocytes) is mediated by autoantibodies. Autoantibodies against membranal and cytoplasmic components of melanocytes were found in the sera of patients with vitiligo and were identified as IgG antibodies (*Moellmann* et al., 1985). Recently, we and others defined tyrosinase, an enzyme which participates in the melanin synthesis, as the autoantigen in vitiligo (*Song* et al., 1994; *Baharav* et al., 1996). Melanoma represents an uncontrolled growth of pigmented cells (melanocytes). The tumour is highly immunogenic and the patients are producing antibodies against the melanoma cells. Since these antibodies react against normal melanocytes, some patients develop vitiligo and are considered to have a better prognosis (*Merimsky* et al., 1994). These relationships between the two diseases have led us to raise the question whether the autoantibodies produced in vitiligo could destroy melanoma cells and serve as a "natural immunotherapy" for melanoma.

Binding of sera from patients with vitiligo to melanoma cells (B-16 murine melanoma cells or M-14 human melanoma) was shown by ELISA studies. Sera derived from patients with diffuse vitiligo yielded the highest titres of anti-melanoma antibodies in comparison to the controls, while patients with localised vitiligo showed lower titre of autoantibodies.

Lysis of tumour melanoma cells in the presence of autoantibodies from patients with vitiligo and complement was demonstrated by proliferation as well as morphological studies and served as the basis for the mice studies.

In vivo studies in which IgG fractions from the sera of patients with diffuse vitiligo were purified on absorption columns and employed for the treatment of melanoma bearing mice. Melanoma metastatic foci were inhibited by 80% (*Fishman* et al., 1993). Additional example for an autoantigen which appears both in melanoma and vitiligo is tyrosinase. Tyrosinase is an enzyme which participates in the process of melanin production in normal melanocytes and melanoma cells. Enzymes are known to be autoantigens in various autoimmune disorders, thus following the detection of anti-tyrosinase antibodies in vitiligo and melanoma, tyrosinase was defined by us as an autoantigen in these condi

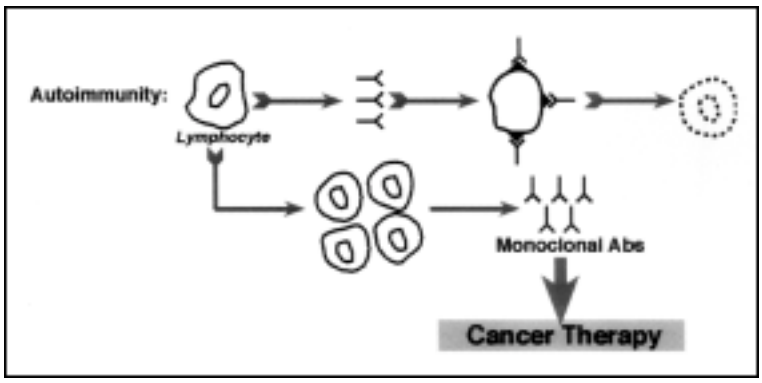

Figure 2: Membranal phospholipids are known to be asymmetrically distributed between the two leaflets of the bi-layer cell membrane. Phosphatydilserine (PS) is localised exclusively in the inner leaflet of the cell membrane of normal cells (Top left). The translocation of PS from the inner to the outer cell membrane is typical to tumour cells which express 7-8 fold more PS on their outer leaflet than normal cells (Top right). Patients with anti-phospholipid syndrome have in their serum anti-phospholipid antibodies including anti-phosphatydilserine antibodies. The hypothesis is that these autoantibodies are capable to bind to tumour cells through the PS.

tions (*Baharav* et al., 1996). In some patients with melanoma the disease is associated with the appearance of "vitiligo-like" white patches on the skin, namely melanoma associated hypo-pigmentation (MAH). In patients with melanoma, those with a metastatic disease showed a higher titre of anti-tyrosinase antibodies in comparison to healthy subjects, while patients with MAH and those with no evidence of disease had similar titres to the control group. The titre of anti-tyrosinase antibodies in patients with metastatic melanoma treated by vaccination with anti-idiotypic antibodies mimicking the high molecular weight melanoma associated antigen, increased following the vaccination and then decreased. High titres of anti-tyrosinase antibodies were detected in patients with diffuse vitiligo in comparison to patients with localised disease and to the healthy control (*Merimsky* et al., 1994).

Mice immunised with tyrosinase, generated a high titre of anti-tyrosinase antibodies and following the inoculation of melanoma cells developed lower number of lung metastases compared to an unvaccinated control group.

These studies confirmed the efficacy of the autoantibodies as cytotoxic against melanoma cells and prompted us to look for additional "pairs" of autoimmune disease and related neoplasm.

Anti-phospholipid syndrome (APLS) and cancer

This pair of conditions consist of various types of cancer cells which express the phospholipid phosphatydilserine on the outer layer of the cell membrane and, the autoimmune condition antiphospholipid syndrome (APS) (Figure 2). Patients with anti-phospholipid syndrome have in their serum anti-phospholipid antibodies including anti-phosphatydilserine antibodies (*Teruhiro* et al., 1991). These pathogenic autoantibodies were shown to induce the clinical manifestations of the disease characterised by thrombocytopenia, thromboembolic recurrent phenomena and repeated foetal loss.

Membrane phospholipids are known to be asymmetrically distributed between the two leaflets of the bi-layer cell membrane. Phosphatydilserine (PS) is

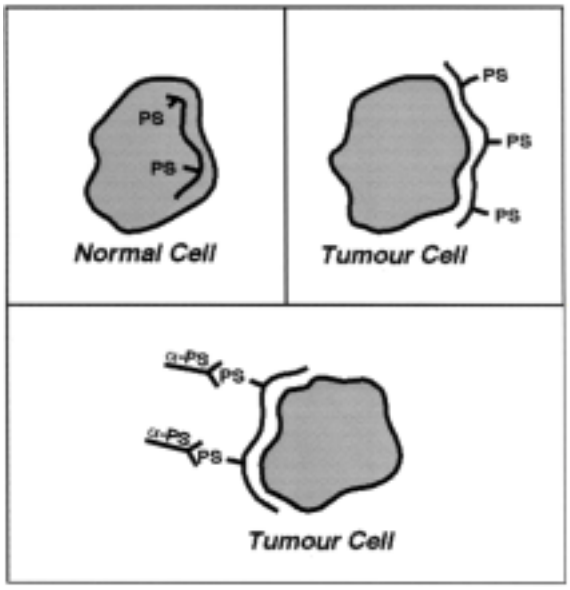

Figure 3: Lymphocytes of patients with autoimmune diseases produce autoantibodies which bind to and destroy normal cells (Top). Lymphocytes from these patients can be expanded and by hybridoma or other techniques, monoclonal antibodies effective against cancer cells can be produced.

localised exclusively in the inner leaflet of the cell membrane. The translocation of PS from the inner to the outer cell membrane is typical to tumour cells which express 7-8 fold more PS on their outer leaflet than normal cells (*Fishman* et al., 1993). We purified the IgG anti-PS antibodies from patients with anti-phospholipid syndrome and examined their *in vivo* efficacy against melanoma tumour cells as was specified above in the studies of vitiligo and melanoma. The anti-PS antibodies exerted inhibitory effect of 76% on the development of lung metastatic foci in the mice inoculated with B-16 melanoma cells. We believe that anti-PS antibodies may be employed in the future for treatment of diverse malignant conditions.

Autoimmune haemolytic anaemia (AIHA) and haematological malignancies

Autoimmune haemolytic anaemia is an autoimmune disease in which the red blood cells (RBC) are destroyed by anti-red blood cell haemolytic autoantibodies. These autoantibodies may be effective against cells in polycythaemia vera (PV), a condition of pseudo-malignant proliferation of RBCs causing clogging of blood vessels and against another proliferative malignant condition - erythro-leukaemia. Binding of serum from a patient with autoimmune haemolytic anaemia to human RBCs or to erythro-leukaemic cells from the murine Friend's cell leukaemia cell line was demonstrated. These kind of autoantibodies eventually can be employed to treat PV and erythro-leukaemia.

Anti-lymphocyte antibodies and lymphoproliferative diseases

In SLE and other systemic autoimmune conditions autoantibodies directed against lymphocytes can be found. These cytotoxic antibodies can be used to treat malignant diseases originated from lymphocytes such as chronic lymphocytic leukaemia, or lymphomas.

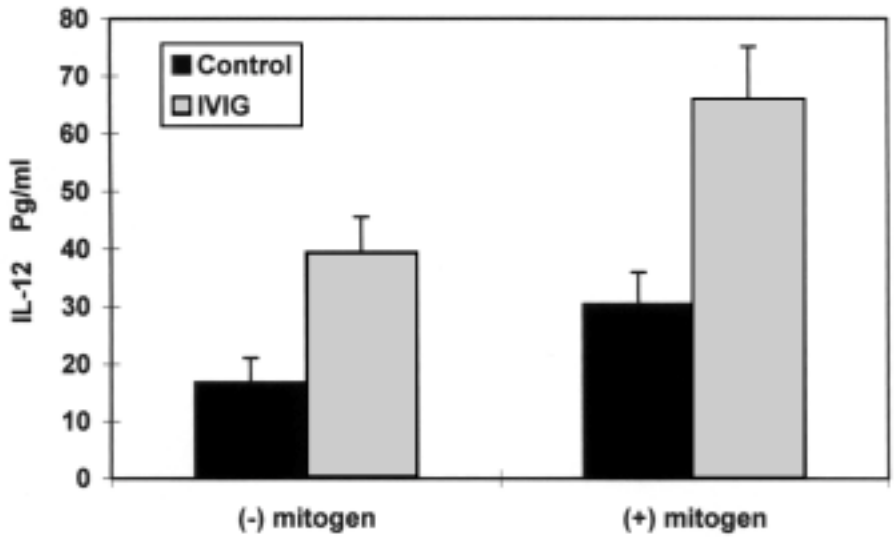

Figure 4: Effect of IVIg (100 μg/ml) on the *in vitro* production of IL-12 by peripheral blood mononuclear cells derived from healthy volunteers. IVIg stimulated the mitogenic induced as well as the spontaneous IL-12 production.

Pemphigus vulgaris and squamous cell carcinoma

The anti-keratinocyte antibodies produced in pemphigus vulgaris are appealing candidates for therapeutic modality to squamous cell carcinoma derived from the skin or other organ origin.

The diversity of autoimmune diseases provides a great source for human performed highly specific autoantibodies which can be used as an effective immunotherapy by themselves. Lymphocytes derived from these patients have the "know- how" to generate *in vitro* monoclonal antibodies which can be implemented for cancer therapy (Figure 3).

UTILISING IVIg, A COMMON TREATMENT IN AUTOIMMUNITY, FOR CANCER THERAPY

IVIg (intravenous immunoglobulin) is the human serum immunoglobulin fraction that is mainly composed of IgG which is prepared from large plasma pools of more than 15,000 healthy blood donors and is suitable for intravenous use. High dose IVIg was first employed to treat patients with immunodeficiencies and later on for patients with diverse autoimmune states. The IVIg was found to affect autoimmune conditions through multifactorial mechanisms (*Kazatchkine*, 1996). These are divided into humoral mechanisms which include Fc blockade by the IVIg, effects on autoantibody binding and production via the idiotypic anti-idiotypic network, prevention of immune complex formation and neutralisation of microbial toxins (*Freitzs* et al., 1991). IVIg also exerts its effects via cellular mechanisms entailing immune modulation of T and B cell number and function, as well as inhibition of anti-inflammatory cytokine production (*Skansen-Saphir* et al., 1994).

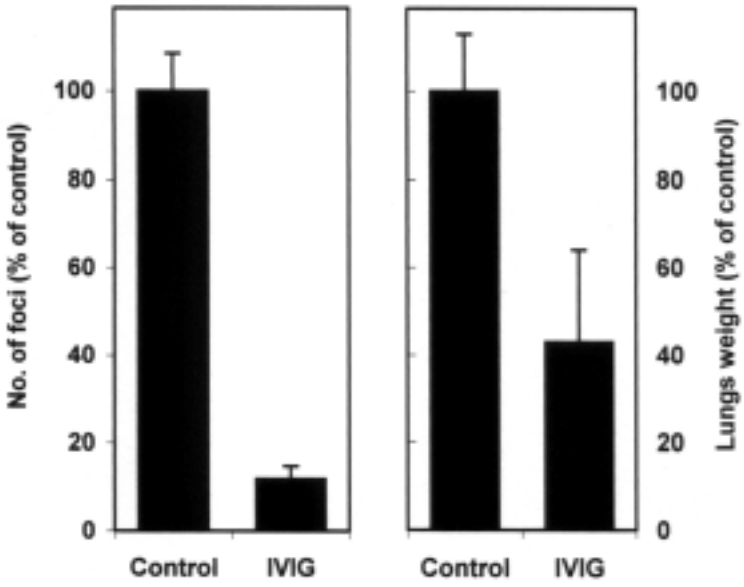

Figure 5: Lung metastatic foci and lung weight of SCID mice, i.v. inoculated with SK-28 human melanoma and treated with IVIg.

Several findings explored recently by our group and others have prompted us to employ IVIg for the treatment of cancer. IVIg was shown to bind to several tumour associated antigens (PSA, CA-125) facilitating its activity as an anti-tumour antibody; it stimulates IL-12 production (Figure 4), a cytokine known to induce anti-cancer activity by activating NK cells and by inducing anti-angiogenic effect (*Hiscox* and *Jiang*, 1997; *Trinchieri*, 1998; *Voest* et al., 1995). Indeed, enhanced NK activity of peripheral blood cells was observed by us and others following incubation with IVIg (*Sgadari* et al., 1996; *Vassilev* et al., 1996) showed that IVIg contains antibodies to a peptide that covers the Arg-Gly-Asp (RGD) sequence which defines the binding site of a variety of adhesive proteins. Thus IVIg may act as an anti-adhesive agent and prevent tumour spread.

This body of evidence led us to carry out a set of *in vivo* experiments which exemplified the efficacy of IVIg as an anti metastatic agent (*Shoenfeld* and *Fishman*, 1999).

The efficacy of treatment with gamma-globulin in murine melanoma was demonstrated by using two models. Its effect (at either high or low doses) was shown by the reduction in the number of lung metastases in mice inoculated with melanoma cells (Figure 5). Furthermore, injecting the gamma-globulin to mice after inoculation of the malignant cells to the foot pad and before amputation of the inoculated limb, was found to prolong survival and to induce 50% cure in the treated mice (Figure 6). The latter model actually mimics the situation in patients in whom the primary tumour is resected and after which treatment is implemented. IVIg was also effective in preventing the development of the lung MCA-105 sarcoma (Figure 6).

As detailed above it seems that the mechanism through which IVIg prevents metastatic spread may be multifactorial and as was detailed above in the

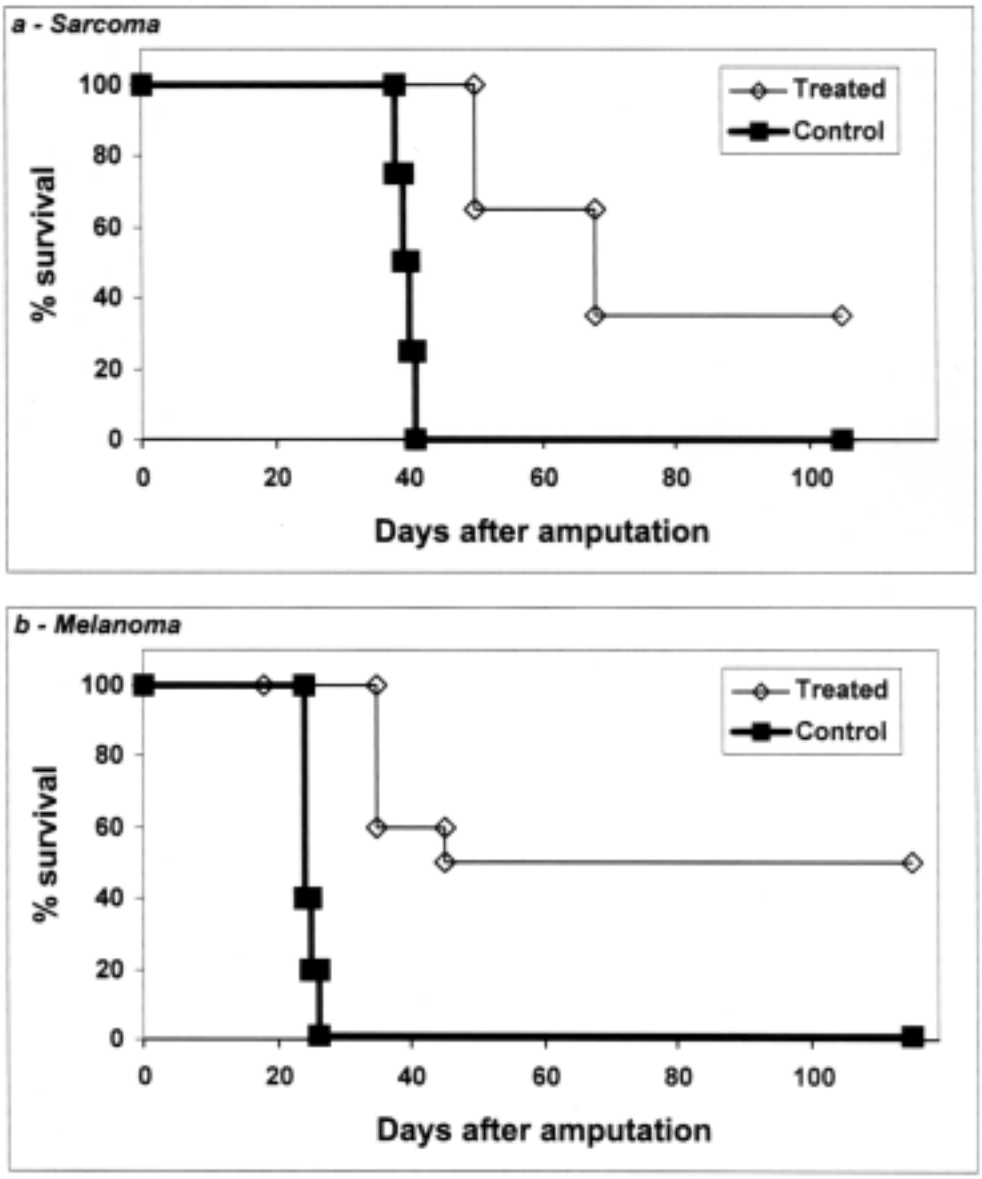

Figure 6: Survival time of mice inoculated with MCA-105 Sarcoma or B-16-F10 melanoma following treatment with IVIg.

paragraph summarising our *in vitro* studies, IVIg practically can affect each step in the process of metastatic spread, from angiogenesis to direct killing (lysis) of the malignant cell.

Thus, IVIg, a classical treatment for autoimmune diseases was found to act as an inhibitor of tumour spread and is currently being tested in clinical trials.

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THE POSSIBLE ROLE OF THE ID-NETWORK IN THE DEVELOPMENT OF LATE ONSET GRAFT-VERSUS-HOST DISEASE AFTER BONE MARROW TRANSPLANTATION: IMPORTANCE OF THE MICROFLORA OF THE BONE MARROW RECIPIENT

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SUMMARY

One of the major complications of allogeneic bone marrow transplantation (BMT) is graft-versus-host disease (GvHD), which is caused by donor type lymphocytes which react against the recipient's tissues.

An important factor which influences GvHD is the recipient's gastrointestinal microflora. This was originally observed in gnotobiotic mice. Infusion of 10^7 H-2 incompatible bone marrow cells into lethally irradiated (9.0 Gy X-rays) conventional mice results in a late onset type GvHD which causes the death of the majority of the recipients during the first two months after BMT. This mortality can be completely prevented if the recipients are germfree mice, or when they are conventional animals which have been subjected to complete gastrointestinal decontamination.

Donor (C57Bl/Rij) and recipient (C3H/Law) mice with different defined microfloras were obtained by using offspring from germfree C3H/Law mothers that were contaminated with the different floras or by using those C3H/Law females to foster hysterectomy-derived C57Bl/Rij new-borns. Studies using these donor and recipient mice resulted in significantly different mortality rates due to GvHD. This observation can be explained by the hypothesis that during gestation and fostering, the developing immune system of new-born animals is modulated by the "experienced" immune system of the dam, resulting in immunological tolerance to flora-components of the (foster-) mothers.

INTRODUCTION

Allogeneic bone marrow transplantation (BMT) is an accepted treatment for many fatal diseases of the haemopoietic system, among them severe aplastic anaemia and leukaemia. Furthermore, patients suffering from fatal hereditary diseases that are associated with a dysfunction of the lymphoid system, like severe combined immunodeficiency, and patients with inherited severe metabolic disorders are being treated with bone marrow grafts.

One of the major complications of allogeneic BMT is graft-versus-host disease (GvHD), which is caused by donor type lymphocytes which react against the recipient's tissues. According to an evaluation of data from 2036 recipients of HLA identical sibling bone marrow transplants reported to the International

Table 1: Composition of the SPF- and Houston-flora

SPF-flora (SPF)	Houston-flora (HF)
Anaerobic microflora: Not defined	**Anaerobic microflora:** Not defined
Aerobic microflora:	**Aerobic microflora:**
Streptococcus faecalis (7173711♣)	*Streptococcus faecium* (7355510♣) *Streptococcus faecium* (7317550♣)
Staphylococcus aureus (6726153♦) *Staphylococcus epidermidis* (6706133♦)	*Staphylococcus xylosus* (6736552♦) S*taphylococcus haemolyticus* (6632171♦)
Escherichia coli (5144572♠)	*Escherichia coli* (5144572♠) *Escherichia coli* (5144532♠) *Proteus mirabilis* (0536000♠) *Proteus mirabilis* (0534000♠)
	Pasteurella pneumotropica (1220000♥)[1]

♣ : Biotype (API 20 Strep; API System, Montalieu-Vercieu, France)
♦ : Biotype (API Staph)
♠ : Biotype (API 20 E)
♥ : Biotype (API 20 NE)
[1] : Isolated from nasal washings only

Bone Marrow Transplant Registry, moderate to severe GvHD occurred in about 45% of these patients. In 48% of them, GvHD was related to their death (*Gale* et al., 1987). The severity of GvHD is influenced by several factors, which include the degree of immunogenetic disparity (*Uphoff* and *Law*, 1958) the number of cells grafted (*van Bekkum*, 1964), the number of T-lymphocytes present in the graft (*van Bekkum*, 1964; 1972), the donor's sex (*Gale* et al., 1987) and the age of the recipient (*Gale* et al., 1987).

MICROFLORA AND GRAFT-VERSUS-HOST DISEASE

Another important factor influencing GvHD is the gastrointestinal microflora of the recipient. This was originally observed in gnotobiotic mice. Infusion of 10^7 H-2 incompatible bone marrow cells into lethally irradiated (9.0 Gy X-rays) conventional mice results in a late onset type GvHD which does not give rise to symptoms until about three weeks after BMT. This disease kills the majority of the recipients during the next two months but those that survive for more than three months seem to have recovered (*van Bekkum* and *de Vries*, 1967; *van Bekkum* et al., 1974). Mortality attributable to this type of GvHD can be completely prevented if the recipients are germfree mice (*van Bekkum* et al., 1974; *Jones* et al., 1971; *Truitt*, 1978) or when they are conventional animals which have been subjected to complete gastrointestinal decontamination by means of orally administered non absorbable antibiotics prior to transplantation (*Truitt*, 1978; *Heit* et al., 1973). These findings in mice suggest that not only histoincompatibility determines the occurrence and severity of GvHD, but that microflora-related factors also are of major importance.

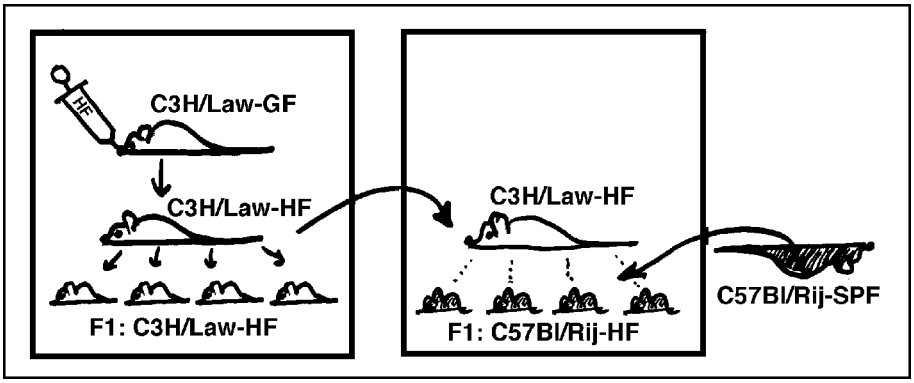

Figure 1: Production of F1 C3H/Law-Hf and F1 C57Bl/Rij-Hf recipient and donor mice.

To study the mechanism, which underlies the influence of the gastrointestinal microflora-components on GvHD, H-2 different donor (C57BL/Rij) and recipient (C3H/Law) mice with a specified pathogen free (SPF) and a conventional microflora were employed (*Heidt*, 1989). For this purpose a conventional microflora was imported from the M.D. Anderson Cancer Institute, Houston, TX, USA, called "Houston flora" (HF), since at that time only SPF animals were being bred in our institute. The composition of the SPF flora and this HF is given in Table 1.

HF bearing C3H/Law mice were obtained by associating germfree C3H/Law breeding pairs with the conventional (HF) flora (Figure 1). Their offspring (C3H/Law-HF) was used as donor or recipient for the different experimental groups. HF bearing C57BL/Rij mice were obtained by foster nursing caesarean derived C57BL/Rij new-borns by C3H/Law-HF mothers (Figure 1).

Before entering the experiment, all mice were kept in Trexler type plastic film isolators to prevent undue association with any other microorganisms. During the experiments, all recipients were housed under conditions of strict reverse isolation in a laminar cross flow isolator to prevent contamination of the animals with any new microorganisms (*van der Waaij* and *Andreas*, 1971). The animals received autoclaved (10 min., 134°C) AM-II food pellets (Hope Farms B.V., Woerden, The Netherlands) and acidified (pH 2.8) sterile drinking water.

According to the microbiological status of the donors and the recipients, there were four different experimental groups. They were: C3H/Law-HF recipients of C57BL/Rij-SPF donor bone marrow (SPF→HF), C3H/Law-HF recipients of C57BL/Rij-HF donor bone marrow (HF→HF), C3H/Law-SPF recipients of C57BL/Rij-SPF donor bone marrow (SPF→SPF), and C3H/Law-SPF recipients of C57BL/Rij-HF donor bone marrow (HF→SPF).

The recipients were lethally (9 Gy) irradiated as a conditioning for BMT. The next day, they were injected i.v. with 10^7 bone marrow cells from C57BL/Rij donor mice. Irradiation of the mice and transplantation of the bone marrow cells were also performed under conditions of strict reverse isolation.

No significant mortality from GvHD occurred in HF→HF, SPF→SPF and HF→SPF recipients ($p>0.10$), but the mortality of SPF→HF recipients was 80% (Figure 2).

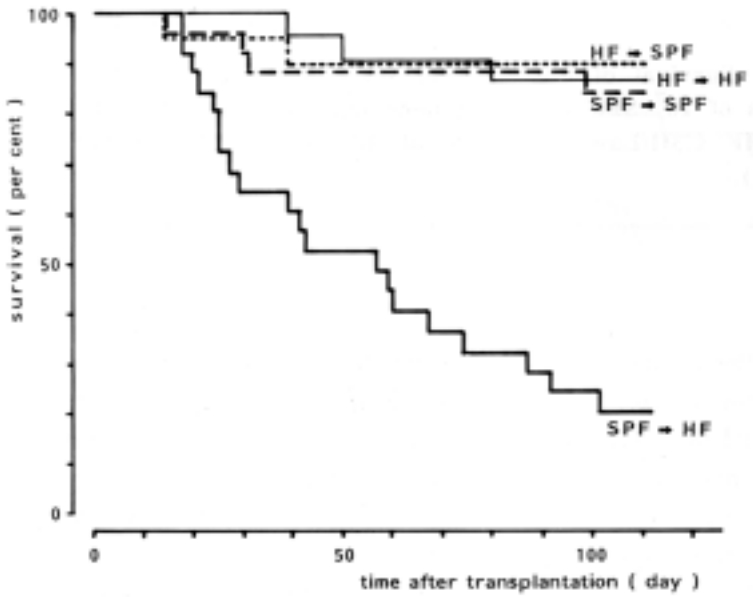

Figure 2: Survival of irradiated (9Gy) C3H/Law recipients of 10^7 C57Bl/Rij bone marrow cells in the different experimental groups (SPF→HF, HF→HF, SPF→SPF, and HF→SPF).

HYPOTHESIS

The difference in survival of the different experimental groups can be explained by a modulating influence of the developing immune system by the "experienced" immune system of the dam (see Figure 3). The ontogenesis of the immune system of these offsprings may have been modulated during gestation by the immune system of their natural mothers. After delivery, the immune system of their foster mothers (the new-borns being transferred immediately after "birth") may have additionally provided "immunologic information about their intestinal microflora". As a result, these foster nursed (HF) offspring may have had a double (suppressive) immunomodulation regarding the SPF microflora as well as to the HF microflora.

During pregnancy, clonal suppression/deletion, regarding HF-components of the mother may have occurred in the foetuses. After birth, those new-borns which became physiologically associated with the HF of the dams may not have responded with IgG B-cells as vigorously as their (originally germfree) mothers did when associated with the HF. Some litters in the first generation of HF offspring may, as they were possibly missing the polyspecific IgM clone due to a vigorous (IgG) response of their mother, still have formed IgG. In general however, they may have "learned" to "tolerate" the same (wide) spectrum of meanwhile autochthonous intestinal bacteria as their mothers.

At the time of bone marrow harvesting for transplantation, the bone marrow of SPF-mice may not have contained conventional B- or T-cells. However, immune-cells potentially reactive to the microflora of the HF-recipients may have been present in the donor cells.

The immune system of the HF donor mice may have become tolerated for the HF during lactation by their HF foster mothers. The immune system of the

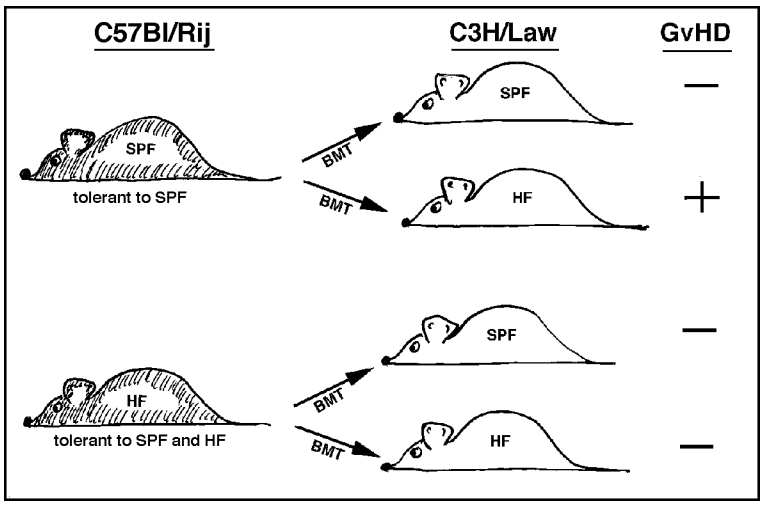

Figure 3: Influence of donor tolerance to different floras on graft-versus host disease (GvHD) after allogeneic bone marrow transplantation (BMT).

C57Bl/Rij-SPF mice was normally tolerated for the SPF microflora during pregnancy as well as during lactation (the latter may only be important for individuals living in a conventional environment).

Indeed we found only significant GvHD in the SPF→HF donor-recipient combination. No GvHD was seen upon HF→SPF and HF→SPF transplantation.

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THE IMPORTANCE OF DONOR MICROFLORA IN LATE-ONSET GRAFT VERSUS HOST DISEASE

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SUMMARY

Late-onset Graft versus Host Disease (LO-GvHD) may occur in lethally irradiated mice after allogeneic bone marrow transplantation. In contrast to acute GvHD, which is caused by T-cells, late onset GvHD is effected by the presence of intestinal microflora in the recipient during the first 40 days after transplantation. LO-GvHD does not occur in germfree or totally decontaminated animals. Based on the results of fundamental studies this review discusses the possible mechanism of LO-GvHD which is characterised by wasting disease and hypoplasia of lymphoid organs. In order to induce LO-GvHD engrafted bone marrow cells need the presence of intestinal microflora in the recipient. In normal situations the autochthonous (self) intestinal microflora, which predominantly consists of anaerobes, induces immuno-tolerance but not immune reactivity within the host. Depending on the degree of similarity with self microflora, non-self i.e. allochthonous microflora may induce immune reactivity instead. Immuno-tolerance can be enhanced by intra peritoneal challenge with autochthonous microflora but not or only little with allochthonous microflora. This review discusses the increase of LO-GvHD in gnotobiotic C3H recipients associated with microflora of C57Bl donors. LO-GvHD was found to the highest level when C57Bl donors in turn had been challenged with their own microflora mice 10 days before bone-marrow harvesting. In conclusion a) BM cells carry “memory” for MF antigens, b) autochthonous-(like) microflora antigens predominantly induce tolerance i.e. are non-immunogenic and c) Recipients colonised with Donor-like microflora are at increased risk for LO-GvHD.

INTRODUCTION

Allogeneic Bone Marrow Transplantation (BMT) following aggressive (sublethal) chemo and or radio-therapy has become a standard procedure in the treatment of certain haematological malignancies. However, regardless optimal donor selection, Graft versus Host Disease (GvHD) may occur as a serious complication after allogeneic BMT. Despite the fact that there are many causes and definitions for GvHD, it is generally circumscribed as immuno-competent, host incompatible, cells i.e. bone marrow, spleen, and/or T-cells that attack lymphoid and epithelial tissues of the host (recipient). Meanwhile, the recipient should be unable to counter-attack the engrafted cells.

Several factors have been found to contribute to GvHD:

a) Major Histocompatibility Complex (MHC) difference between donor and recipient
b) the type of cells engrafted e.g. purified T-cells, spleen cells in combination with BM cells
c) the number of cells engrafted
d) the intestinal microflora

Several precautions are generally taken in order to prevent GvHD:

a) donor and recipient are matched as much as possible according their MHC antigens; i.e. HLA in man and H-2 in mice
b) the bone marrow graft is depleted from T-cells if present
c) patients are totally decontaminated and nursed under a germfree conditional regimen
d) patients receive short or long-term GvHD immunosuppressive therapy

Due to these additional regimens GvHD has decreased and is no longer a serious threat after allogeneic BMT. Total decontamination, and thus the need for strict barrier nursing, is not only effective in preventing non-viral infections but also GvHD (*van der Waaij* and *Heidt*, 1986; *Beelen* et al., 1992). However, some negative aspects still remain; e.g. strict barrier nursing procedures are laborious and therefore very expensive, and implicate strong psychological stress for the patient. Whereas immunosuppressive therapy is effective against GvHD, patients treated as such are prone to viral infections and tumour development.

One of the questions worth investigating is why recipient intestinal microflora in a way induces or aggravates GvHD. As noted, GvHD can be mitigated by total decontamination. However, if the question can be answered how the microflora is instrumental in GvHD, total decontamination combined with expensive high care and germfree strict-barrier nursing would no longer be necessary. In this way the duration of GvHD suppressive therapy might be shortened, if still necessary, implicating a lower risk for viral infections to occur. This may additionally implicate maintenance of the potentially beneficial immuno-stimulating effects by components of the gut microflora as well as a better control of colonisation of the intestinal tract by potential pathogenic bacteria and fungi.

HISTORY

In the late sixties and early seventies Van Bekkum et al. found a strong correlation between intestinal microflora (I-MF) and GvHD in mice (*van Bekkum* et al., 1974; *van Bekkum* and *Knaan*, 1977). MF-associated GvHD, at first named 'secondary disease', was found when lethally irradiated conventional inbred mice were engrafted with normal, T-cell negative, BM (T^-BM) from H-2 incompatible (allochthonous) donors. MF associated GvHD was found to differ from acute GvHD, found after engraftment of BM in combination with spleen or T-cells (T^+BM). Important differences, which were seen between T^-BM and T^+BM engrafted mice, were:

a) mortality in T^+BM recipients was 100% whereas only a part of the recipient group died when engrafted T^-BM
b) mice engrafted with T^+BM died within 21 days whereas T^-BM mice died between 28 until 100 days after BMT
c) signs and symptoms of GvHD were totally absent in germfree or totally decontaminated T^-BM engrafted mice
d) mortality was delayed but still 100% in germfree or totally decontaminated recipients engrafted with T^+BM.

Because of the delayed occurrence, MF associated GvHD is named Late Onset GvHD (LO-GvHD). LO-GvHD in mice is characterised histologically by aplasia of lymphoid organs e.g. thymus, spleen and lymph nodes and destruction of epithelial tissues e.g. skin, and gut. Clinically, mice suffering from LO-GvHD show diarrhoea, progressive wasting, and a ruffled fur. In contrast to T-cell mediated acute GvHD, LO-GvHD is not always fatal. Additional investigations have shown that total mitigation of LO-GvHD remains when mice are maintained bacteria-free i.e. germfree until 40 days after BMT by which time they can be conventionalised without adverse effects (*van Bekkum* and *Knaan*, 1977). This window phase of 40 days found in mice may exist in man as well. These observations have been one of the reasons why patients nowadays receive total decontamination initially after BMT for a restricted period of several weeks. Indeed, there is increasing evidence that total decontamination reduces GvHD in man (*Beelen* et al, 1992; *Heidt*, 1989; *Vossen* et al., 1990).

Despite the fact that elimination of potential pathogenic bacteria e.g. *Enterobacteriaceae* and *Pseudomonadaceae* during the leukopenic phase has been found to reduce the number of infections, septic periods, and subsequently death, no direct correlation has been found between LO-GvHD and these bacteria as infectious agents (*Veenendaal* et al., 1988; *Vossen* et al., 1990). Instead, it has been postulated that bacteria in the intestinal tract, particularly Gram-negative rods, induce antibodies that cross react with tissue antigens and subsequently cause LO-GvHD (*van Bekkum* and *Knaan*, 1977; *van der Waaij* and *Heidt*, 1986). However, there is ample evidence that Gram-negative rods e.g. *Enterobacteriaceae* are of minor importance if any in the pathogenesis of LO-GvHD (*Heidt*, 1989; *Veenendaal* et al., 1988; 1990; *Vossen* et al., 1990; *Veenendaal*, 1995) and if so their role may be limited to a non-specific immuno-modulation by lipopolysaccharide (LPS) i.e. endotoxin (*Moore* et al., 1987a; 1987b).

The conditions needed for LO-GvHD to occur are fourfold:

a) recipients should either carry a conventional or a diverse but well developed SPF microflora,
b) should receive lethal irradiation prior to BMT,
c) BMT should be carried out over a total MHC (H-2) barrier, and
d) some sort of interaction has to take place between intestinal microflora antigens and engrafted BM cells e.g. NK-cells or B-cells.

In mice, the latter (d) apparently takes place during the window phase lasting until 40 days after BMT causing LO-GvHD.

The window phase gives proof that I-MF plays a central role in the pathogenesis of LO-GvHD. Thus MF associated LO-GvHD in fact appears to be Graft versus Microbial Flora Disease. An important feature of LO-GvHD is the absence of proper T-cell function in combination with dysplasia of the thymus. This finding has similarities to thymusless nude mice. In fact nude mice also suffer from runting disease if they are maintained under conventional conditions. There is a main difference, however, between nude mice and LO-GvHD mice. Whereas nude mice already have a deficient T-cell system from the start, T-cell deficiency develops during LO-GvHD after BMT. This difference is subject for discussion. The critical window phase of 40 days, during which mice are prone to suffer from LO-GvHD, generates many questions e.g.:

a) What type of interaction takes place between donor BM-cells and recipient-MF which eventually leads to LO-GvHD

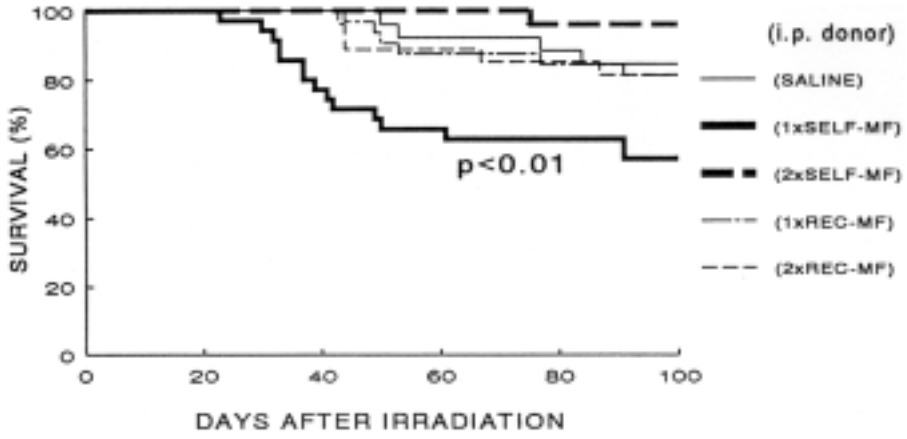

Figure 1: Survival rates of lethally irradiated (9 Gy) C3H/He (H-2k) recipients engrafted with 10^7 nucleated BM cells from C57Bl/6J (H-2b) donors 19-100 days after BMT. C57Bl Donors had been pretreated by i.p. injection either with saline, washed faecal flora of their own (SELF-MF) or C3 recipients (RECIP-MF). I.p. injection was performed on day -10 for single injection and day -38 and -10 for repeated injection. The number of recipients at day 19 were: group A (2x saline) n=26, group B (1x SELF-MF) n=35, group C (2x SELF-MF) n=25, group. D (1x RECIP-MF) n=32, and group E (2x RECIP-MF) n=26. Mortality in group B (1x SELF-MF) was significantly the highest ($p<0.01$) compared to all other groups (Kaplan-Meier analysis).

b) Is there any evidence for donor BM-cells with specific affinity for (components of) the recipient-MF
c) Is there any correlation between composition of the donor-MF, the recipient-MF, and LO-GvHD
d) Is (LO-)GvHD generated by some sort of key component in the recipient-MF
e) Should an immunological classification system of intestinal microflora be developed to explain, predict, and make it possible to prevent LO-GvHD in the future

DONOR MICROFLORA AND LO-GvHD

Experiments in mice have shown a close interaction between intestinal (microflora) antigens and the BM compartment (*Alley* et al., 1986; *Veenendaal*, 1995). Even microflora modulation, induced by oral treatment with small spectrum antibiotics as used for selective decontamination, has been found to influence the composition of the BM (*Goris* et al., 1985; 1986). Additionally, an enhancing effect on LO-GvHD in C3H recipient mice has been observed when C57Bl donors are given selective decontamination for two weeks prior to BMT (*Veenendaal* et al., 1988). This result leads to the postulation that engrafted BM cells carry certain 'memory' for MF-antigens, present in the recipient, which is activated by oral treatment with non-absorbable small spectrum antibiotics. It seems most likely that this effect is caused by bacterial antigens, which could for example be released in the intestinal lumen during antibiotic treatment upon bacterial disintegration, pass through the intestinal mucous and after some time reach the circulation. Experiments in which

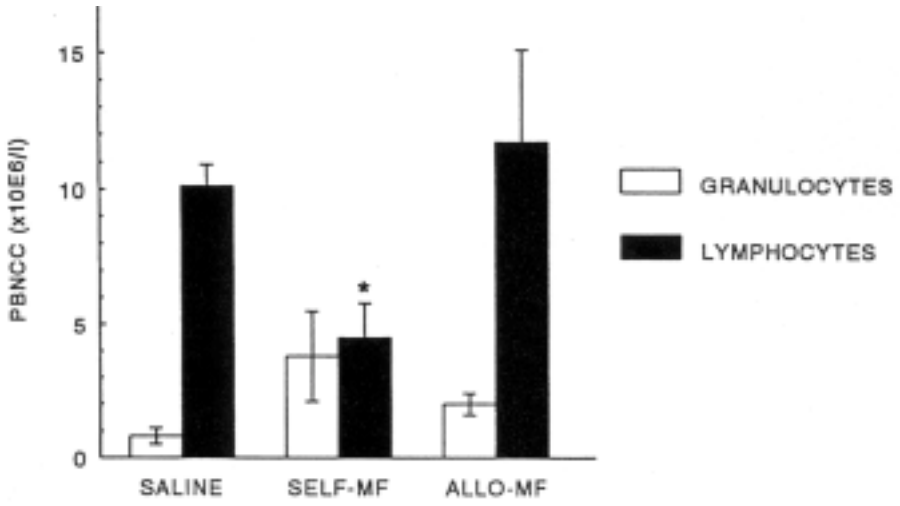

Figure 2: Peripheral blood nucleated cell count (PBNCC); granulocytes and lymphocytes in C57Bl/6J mice 10 days after i.p. injection with 0.5 ml washed faeces from the B6-strain (SELF-MF) (n=8) or C3H/He mice (ALLO-MF) (n=7). Control donors (n=7) were injected with saline only. *: $p<0.05$

C57Bl donors were challenged intra peritoneal with either their own MF or that of the future C3H recipients showed that LO-GvHD increased significantly when BM donors received one *single* intra-peritoneal (i.p.) injection with their own MF (SELF-MF) 10 days before BMT (*Veenendaal*, 1995). In some way these animals were given an artificial form of enhanced translocation of autochthonous microflora. This effect disappeared when donors were i.p. injected *twice* with SELF-MF respectively 38 and 10 days before BMT. LO-GvHD was found at the lowest level in this group. Parenteral challenge of BM donor animals with recipient-MF (NON-SELF-MF) surprisingly did not influence LO-GvHD compared to saline injected control donors (Figure 1). Thus LO-GvHD in these studies was not only affected by the recipient MF. Instead, the donor MF appeared to be even more important. This effect was found in a mouse model in which LO-GvHD was present at a low baseline level giving a mortality rate of approximately 20%.

INTERACTIONS BETWEEN IMMUNE SYSTEM AND MICROFLORA

Different functional changes not only occur in the BM upon parenteral challenge with SELF-MF antigens compared to NON-SELF-MF but also in the peripheral blood and the spleen. Figure 2 shows that a single i.p. injection of C57Bl mice with SELF-MF decreased the number of lymphocytes in favour of granulocytes as compared to i.p. injection with NON-SELF-MF from C3H mice. Figure 3 shows that spleen enlargement in mice may occur at a higher level after a single i.p. injection with NON-SELF-MF as compared to SELF-MF. On the other hand the spleen size is normalised or not effected anymore after

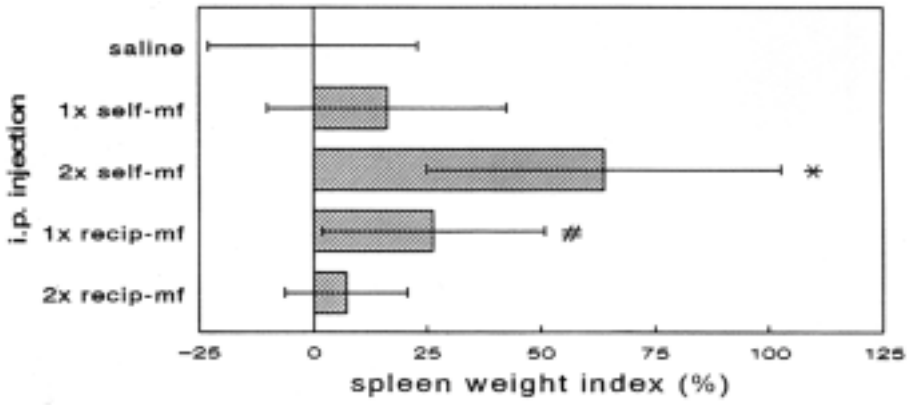

Figure 3: Spleen weight index in C57Bl/6J mice i.p. injected either with saline (n=7), 1x SELF-MF (n=13), 1x RECIP-MF (n=5), 2x SELF-MF (n=12), or 2x RECIP-MF (n=10). I.p. injection was performed on day -10 for single injection and day -38 and -10 for repeated injection. *: $p<0.01$ compared to all other groups; #: $p<0.05$ compared to saline injected animals.

repeated i.p. injection with NON-SELF-MF, whereas the spleen size further increases after repeated i.p. injection with SELF-MF. I.p. injection with SELF-MF and NON-SELF-MF both increase the level of total IgM in serum whereas the level of IgA and IgG in serum remains unaffected (*Veenendaal*, 1995). The same has been found for MF-specific IgM antibodies. However, MF specific IgM antibodies has been found at a significantly higher lever against SELF-MF as compared to NON-SELF-MF originated from C3H mice.

These data implicate an immunological difference between SELF-MF and NON-SELF-MF in the mouse i.e. C57Bl (B6) and C3H (C3) mice. I.p. challenge with either microflora does not induce IgG or IgA seroconversion in B6 mice. Instead, IgM may be the most important regulating immunoglobulin in the immune response against microflora antigens.

Since no difference was found in the number of viable aerobic bacteria present in either MF (Table 1), the bacterial fraction, which has to be held responsible for the different reactions described above, must be looked for within the composition of the highly concentrated anaerobes. Micromorphological examination revealed a difference in this fraction in either MF. This indirectly indicates that LO-GvHD might be associated with differences in the level of (immuno-)regulation between "SELF" (autochthonous) MF and "NON-SELF" (allochthonous) MF; particularly concerning the highly concentrated obligate anaerobes. Herein SELF-MF antigens predominantly may induce immuno-tolerance. When these bacteria 'attack' the host outset their normal habitat e.g. extra-intestinal more primitive immune reactions are the only defence against it. This explains why spleen enlargement further increased upon a second i.p. challenge with SELF-MF but not with NON-SELF-MF.

Immuno-tolerance apparently is important for the symbiosis between the host and its MF. Bacteria may only be able to survive within the intestinal lumen when there is no specific (mucosal) immunity. Lack of mucosal immunity may either be due to a gap in the immune repertoire or due to active suppression by antigen specific suppresser

Table 1: Analysis of pooled faeces from B6 and C3 Mice

Microorganism	B6 (n=5)	C3 (n=5)
E. coli (API:5144552)	1.6×10^3	2.0×10^3
Prot. mirabilis (API:0536000)	2.0×10^4	2.5×10^4
Enterococcus spp.	5.0×10^4	3.0×10^4
Staph. aureus	1.0×10^4	1.5×10^4
Bacillus spp.*	ND**	ND
obligate anaerobic bacteria***	10^{11}	10^{11}

Data represent concentrations in microorganisms/g. faeces
*: Qualitative aerobic culturing only
**: Microscopic analysis by eye revealed a morphological difference between highly concentrated obligate anaerobic fractions in B6 and C3 faeces.
***: ND=not determined

cells. By selecting bacteria that are, or become, immunologically inert, the host will prevent itself from undesirable immune reactions; e.g. antibodies which fix complement and/or cross-react with host type antigens and cause respectively inflammation or autoreactivity.

General suppression of specific immunity against SELF-MF is likely to be compensated by stimulation of the innate immune system i.e. granulocytosis and monocytosis. In the absence of IgG and IgA, the isolated IgM antibody response upon i.p. challenge may indicate the absence of SELF-MF antigen specific Th2-cell mediated immunity. On the other hand the existence and increase of IgM antibodies may also point at the presence of an idiotype anti-idiotype network which physiologically regulates MF-antigens.

It remains speculative whether, and if so how, induction of immuno-tolerance exists or more likely is enhanced inside the BM compartment upon i.p. challenge with SELF-MF. Possibly during live the host and it's MF live in symbiosis. For reasons of necessary immunological peace with SELF-MF the host only generates an innate (primitive) immune reaction, e.g. granulocytes and macrophages and/or IgM, together with a non-T-cell mediated systemic immuno-suppression e.g. by natural killer or suppresser (NK) cells. These reactions may constantly take place upon physiological translocation of SELF-MF antigens. "Tolerance" mediated by NK cells may either be local in the gut associated lymphoid tissue (GALT) or systemic e.g. at the bone marrow level. In the latter, large granular lymphocytes (LGLs) may function as such. During live, MF and immune system thus are in an equilibrium of "tolerance". However, if during (artificially) enhanced translocation of MF, e.g. by i.p. challenge, MF-antigens "escape" from the local immune defence by the GALT and will be presented at peripheral sites e.g. spleen and bone marrow. When this happens the innate defence (granulopoiesis and myelopoiesis) is stimulated as well as suppression (=tolerance). Subsequently a new equilibrium is formed which supplies adequate defence without systemic stimulation e.g. of BM upon a second artificial enhanced translocation. If true, this explains why repeated i.p. injection of donors with SELF-MF did not change or even slightly mitigated LO-GvHD in C3 recipients shown in Figure 1.

MF that generates immuno-tolerance over a wide spectrum of MF antigens, like the SELF-MF in B6 mice, may be

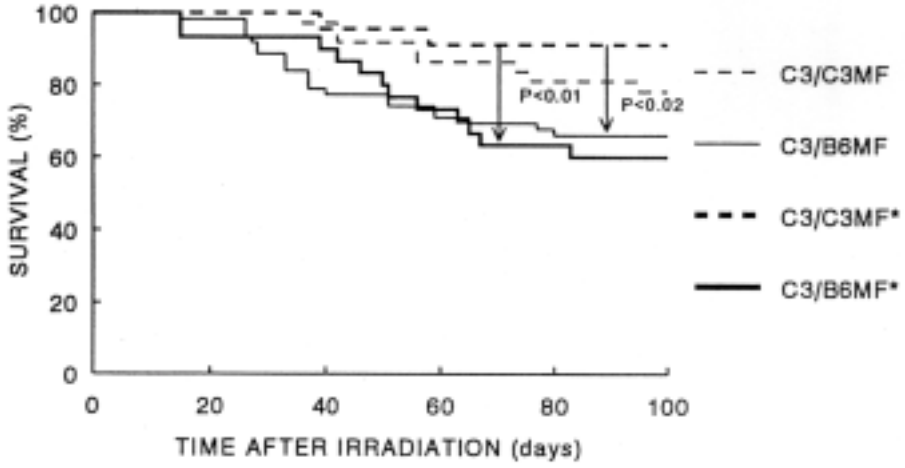

Figure 4: Survival of irradiated (9 Gy) gnotobiotic C3H/He (C3) (H-2k) recipients engrafted with BM from C57Bl/6J (B6) (H-2b) donors. Recipients were the first generation offspring ex-germfree mice associated with murine microflora either from SPF-C3 mice (C3/C3MF) or SPF-B6 mice (C3/B6MF). B6 donors were i.p. injected either with their own B6-MF (i.p./B6) or with saline (-/B6) 10 days prior to BMT. Numbers/group: C3/C3MF (n=37); C3/B6MF (n=62); C3/C3MF* (n=23); C3/B6MF* (n=30). *: B6 donor i.p. with B6MF.

an important tool to predict LO-GvHD. Herein lymphocyte reduction 10 days after a single i.p. challenge with SELF-MF may implicate induction of immuno-suppression or enhancement of existing tolerance. It seems plausible that BM, harvested from B6 mice at this point, increases LO-GvHD in C3 recipients by inducing immuno-suppression; i.e. hypoplasia of lymphoid tissues. This reaction will then be enhanced by immuno-tolerant MF-antigens in the recipient.

As mentioned earlier, LO-GvHD can only occur when engrafted BM-cells face the presence of MF in the recipient. Theoretically, engrafted BM-cells may interact with remaining but not dividing host immune cells with memory for recipient MF-antigens during proliferation and differentiation which takes place during repopulating of the host. However, this may not be very likely since LO-GvHD is prevented by total decontamination of the recipient even if it is started only shortly before BMT.

Based on the findings discussed, it can be postulated that LO-GvHD is to be expected predominantly in a) recipients carrying a donor-like microflora and b) if the donor's MF has recently been modified e.g. by an infectious disease and antibiotic treatment.

MATCHING DONOR AND RECIPIENT MICROBIAL FLORA AND LO-GvHD

We have found that LO-GvHD increases when C3 recipients carry a B6 donor like MF (*Veenedaal* et al., 1990; *Veenendaal*, 1995). The recipients used in this study were the first generation offspring from ex-germfree C3 mice associated either with original C3 or B6-MF and were maintained in separate 'germfree' isolators. The results shown in Figure 4 strongly suggest that recipients, which carry an intestinal microflora which is (partly) homologous

to that of the donor, are at increased risk for LO-GvHD. Since the homologous MF used in this study was original donor type, the donor and particularly its BM may have contained memory for immuno-tolerance against this MF. Again, this "memory" might have been activated to a higher level by a single i.p. injection of donor mice with SELF-MF 10 days before BM harvesting. If this assumption is correct, this feature is of key importance for LO-GvHD to develop.

The question remains, however, which type of BM cells, with apparent immuno-suppressive memory for MF antigens, are involved in the pathogenesis of LO-GvHD. Part of the mechanism that induces LO-GvHD may be an interaction with recipient-MF which has great similarity with the type of immuno-regulation of self-MF antigens. Natural-Killer (NK) and -Suppresser (NS) cells play a profound immuno-regulating role in the bone marrow. Moreover, NK as well as NK-like so-called Large Granular Lymphocytes in bone marrow have been described to play a role in the immunosuppressive phase of GvHD, like LO-GvHD (*Guillen* et al., 1986; *Imamura* et al., 1988). A second and certainly not less important function of NK cells concerns their role in the mucosal immune system (*Brandtzaeg* et al., 1988; 1990). Besides NK or NK-like cells, which are likely to regulate the BM response against intestinal microflora antigens, a regulatory role of anti-idiotypic antibodies i.e. an idiotype anti-idiotype B-cell network should not be excluded on the forehand. However, so far no evidence is available that suggests such a regulatory mechanism in LO-GvHD.

PERSPECTIVES IN MICROFLORA ASSOCIATED GvHD

Despite evidence that genetic differences between donor and recipient are important, LO-GvHD predominantly appears to be caused by the composition of the microflora of both. The precise mechanism of this, in fact Graft versus Microbial Flora, phenomenon remains an enigma. More research is needed to confirm the postulation that NK cells or large granular lymphocytes (LGL) in the bone marrow are indeed instrumental in the development of LO-GvHD. Isolation of NK cells or LGLs from the bone marrow may enable *in vitro* stimulation experiments with related and unrelated whole MFs or with fractions of these. Investigating delayed type hypersensitivity (DTH) responses against MF or MF-fractions by NK-cells may be an alternative for measuring differences in cellular reactivity against MFs of donor and recipient.

B6 donor mice injected with SELF-MF 10 days prior to BMT and C3 gnotobiotic recipient mice associated with the B6 donor MF may serve as a suitable animal model for *in vivo* experiments. This model may also serve for adoptive transfer studies and for kinetic studies with regard to the development of GvH reactions in lymphoid organs like thymus, spleen and bone marrow, as well as non-lymphoid tissue e.g. intestines, and skin. The model may also be used for time/dose finding studies in which not only donors but also recipients are parenterally challenged with different doses of microflora at different times respectively before and after BMT. Cytokines e.g. IFNgamma, and TNF alpha should then be measured during the follow-up after BMT and GvHD in order to collect additional information on the possible subsets of cells, e.g. NK cells and macrophages, that might be involved in the ontogeny of GvHD.

Finally, a simplified classification of

intestinal microflora into related and non-related is functional for discussing LO-GvHD in inbred mice. However, this simplification is difficult to transpose to clinical BMT in which individual patients each may carry a different intestinal microflora (*Apperloo-Renkema* et al., 1992; *Jansen* et al., 1993; *Meijer-Severs* and *van Santen*, 1986). *Apperloo-Renkema* et al. (1992) demonstrated a new technique of microflora analysis which combines quantitative indirect immunofluorescence with digital image analysis. This so called immuno(micro)morphometrical analysis on stool specimens combined with serum, sampled from donors and recipients before and after BMT, may be of great future interest with regard to either predicting risks or monitoring development of GvHD after clinical BMT. An alternative for immuno-(micro)morphometry, may be analysis of faeces and serum by using the fluorescence activated cell sorter, a method described by *van der Waaij* and colleagues (1994). Immuno(micro)morphometry as well as FACS analysis of faeces and serum are easy to apply in human BMT.

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

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

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

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

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

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

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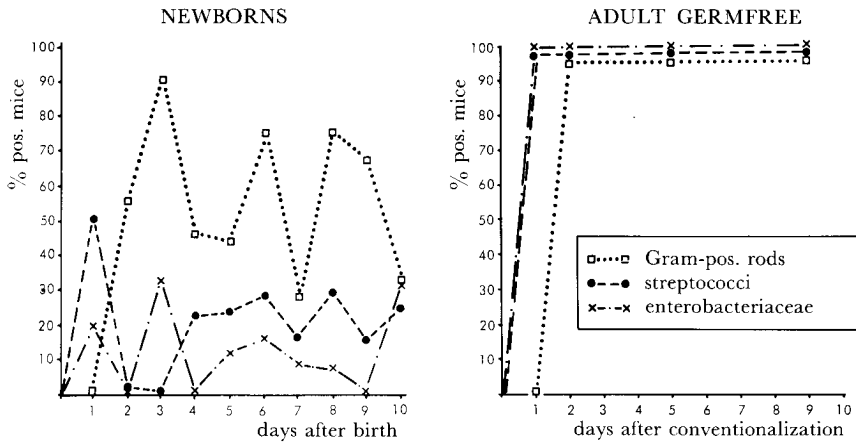


Figure 1: Development of faecal microflora upon conventionalisation of new-born and adult euthymic mice (*van der Waaij*, 1968).

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

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

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

Relevant conclusion:

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

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

The contamination source:

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

The nutritional environment:

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

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

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

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

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

Relevant conclusions:

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

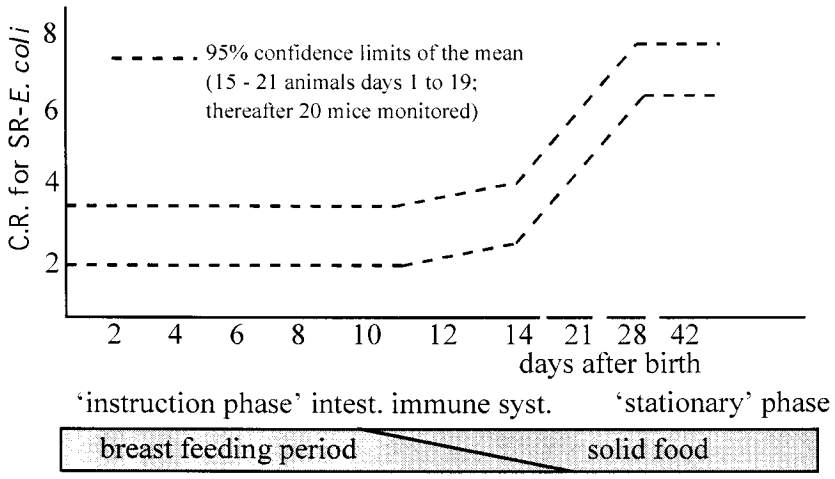


Figure 2: The development of the colonisation resistance for SR-E.coli of the digestive tract since birth (*Heidt* and *van der Waaij*, 1979).

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

THE DEVELOPMENT OF AN INTESTINAL MICROFLORA IN CONGENITALLY ATHYMIC MICE

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

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

Relevant conclusions:

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

[1] Specific pathogen free

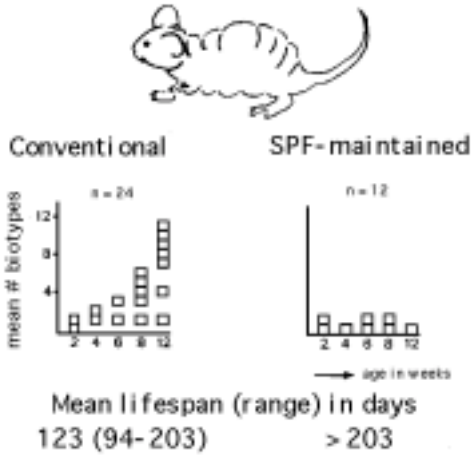


Figure 3: Development of intestinal CR in congenitally athymic mice (*van der Waaij*, 1981).

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

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

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

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

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

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

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

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

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

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

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

Perhaps both factors were involved as:

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

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

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

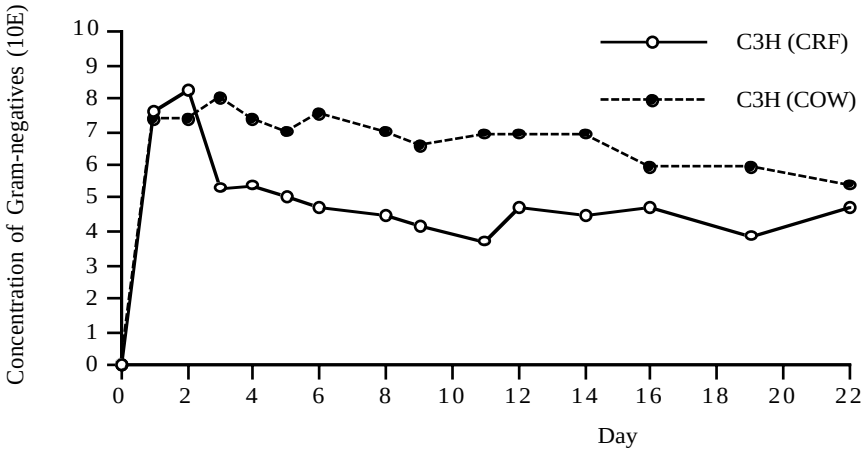


Figure 5: Concentration of *E. coli* in the faeces following oral association with cow faeces in adult ex-germfree mice (*Geertsema* et al., 1990).

might become apparent in these baby mice.

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

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

Relevant conclusions:

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

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

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

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

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

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

The B1-cell system and the (T-cell

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

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

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

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

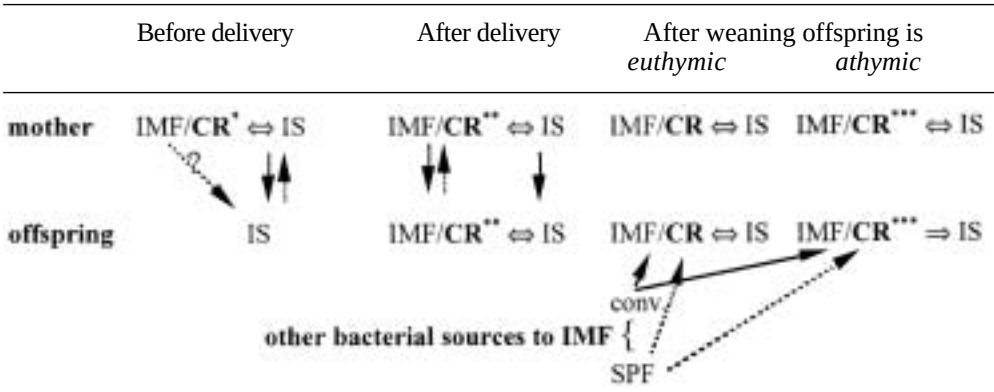
If IgG/IgA plays a role in the control of IMF, it may make survival of bacteria at the site difficult. The bacteria in question may get selectively 'suppressed' and thus in disadvantaged position in comparison with bacteria with no antibodies coating them.

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

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

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

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



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

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

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

IS : Immune system

PROPOSED WORKING HYPOTHESIS

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

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

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

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

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

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

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

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

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

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

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OLD HERBORN UNIVERSITY SEMINAR ON POLYSPECIFIC IMMUNOGLOBULINS, THEIR POSSIBLE ROLE IN THE NORMAL (PHYSIOLOGICAL) CLEARANCE OF MICROORGANISMS AND TISSUE FRAGMENTS: MINUTES AND REVIEW OF THE DISCUSSION

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NATURAL IGA: EFFECT ON TRANSLOCATION

When the intestinal tract of a newborn child is colonised by bacteria, the production of IgA's in the gut lumen is profound. Currently, the specificity of these IgA's is unknown. Studies in mice mono-associated with segmented filamentous bacteria (SFB) suggest that, although the IgA response to this bacterium is very high, little or none of this IgA is specific for the SFB (Gram-positive bacteria that do not produce lipopolysaccharides). How IgA production is stimulated is not known. Probably, IgA induction does not occur through stimulation of class II enterocytes and local release of IgA's by T-cells since most are of the γδ-type, not αβ-type, making a link between class II lymphocytes and T-cells unlikely.

Comparison of IgA responses in mice monoassociated with a variety of different bacteria indicate that the production of specific IgA's (measured against bacterial lysates on slides) does not correlate with the 'total' IgA response. Different species of bacteria each result in different responses and the mouse strain does not affect the response pattern. Furthermore, the level of specific IgA's that is produced does not seem to correlate in any way with the translocation rates determined for these bacteria (measured as the number of bacteria that can be cultured from spleen or mesenteric lymph nodes). Against *Oochromobactrum anthropi*, for example, no specific IgA's are produced, and translocation can be demonstrated. A non-invasive mutant of *Listeria monocytogenes*, on the other hand, is confronted with high levels of specific IgA's and still translocates at high rates. The translocation of *Morganella morganii*, however, is completely prevented once the production of specific IgA's is induced. No general conclusions can be drawn at this moment concerning the level of the specific IgA response and the translocation of the bacterium. One should however bear in mind that when measured as described above, translocation over the intestinal lining may in fact occur at high rates, but rapid clearance of the bacteria in the lamina propria can result in low numbers of culturable cells from peripheral tissues as spleen and mesenteric lymph nodes.

Additional experiments may include investigations on the effect of specific IgA's on injected antigen. The induction of oral tolerance could be used to determine the levels of specific IgA directed towards the antigen versus the level of natural IgA in a culture independent way.

A discussion was raised considering the interpretation of experimental data on specific / non-specific / natural or total and polyspecific and the interpretation of cross-reactivity in this. It was suggested that cross reactivity and specificity are blurred by affinity, which is the result of a physico-chemical relationship between two molecules. Description of affinity, and therefor specificity, in terms of absolute values for one molecule alone are not necessarily meaningful.

The point was made that physiologically meaningful translocation may also involve the translocation of nucleic acids or antigens only. Also, intracellular bacteria cannot be reached by IgA. This complicates the issue even further.

The properties of the individual bacteria are likely more determinative to translocation then the level of IgA production in the lumen. Still, specific IgA's are necessary for the prevention of translocation of *M. morgani*, as was demonstrated in an experiment using mice monoassociated with SFB (high non-specific IgA response) which were superinfected with *M. morgani*. As in germ free-mice, the translocation of *M. morgani* was stopped completely upon the production of specific IgA's against this bacterium. Currently, the bacterial surface antigens with which these specific IgA's interact are not known.

Both B1 and B2 cells may be involved in IgA production. Serum IgA's in mice monoassociated with *O. anthropii* were 100% a type and therefor of B1 origin, whereas a superinfection with *M. morgani* resulted in serum IgA levels that were for 61% of the B2 derived b type.

The time it takes for SFB to colonise the caecum of new-born mice is shortened if the mother is immunocompromised. The levels to which colonisation occurs and the prevalence is affected by the fact whether the offspring is immunocompromised or not. It is therefrom inferred that the IgA's of both the mother and the offspring are important for the composition of the flora and its rate of establishment.

Comparative studies on breast fed children of Pakistan and Sweden indicate that colostral IgA's, from which Pakistani children are deprived, may have a dramatic effect on the turnover of different enteric bacterial strains in the intestinal flora. Frequent, but temporary colonisation with different enteric strains is characteristic for intestinal flora's in Pakistani children, which face higher exposure to these bacteria than do Swedish children. In Pakistani children, the translocation is often higher than what the immune system can handle, resulting in relatively high levels of infant mortality as a result of sepsis (1%).

NUTRITION AND GALT EVOLUTION

The question raised was whether there is a relationship between nutritional status of the mother, the properties of the breast milk and the survival of children. At present, there is no knowledge whether the milk of malnourished mothers is of reduced quality. If an analogy exists with the nutritional status of the mother and the quality of the foetus, one might expect there is no such an effect.

However, the foetal gut is getting

shape after 6 weeks and is formed by endodermic folding. The gut associated lymphoid tissue (GALT) of the foetus starts developing very soon after that. Functions begin to pick up rapidly. Hormonal influences are measurable at 17-20 weeks of development. It has been determined in epidemiological studies that children may experience diseases later in life (at the age of 40) at higher instances in the case of maternal malnutrition in week 10-20.

It seems that nucleotides play a key role in the further development of newborns. Especially nucleotides, which cannot be synthesised *de novo*, are necessary for proper growth of all proliferating tissues, including gut epithelium, cells of the central nervous system as well as cells of the immune system. Considering that 1 mitoses requires 10^9 nucleotides, and assuming an incorporation efficiency of only 5%, a growing child may need up to 450-700 mg of nucleotides per day. Cow milk contains less than 1 mg of nucleotides per litre. Human breast milk, on the other hand, contains 20-70 mg of nucleotides per litre. Specifically in developing countries, children may suffer serious nucleotide deficiency resulting in retarded growth of the nervous system and the GALT. In fact, nucleotide supplements in milk can decrease the incidence of gut infections in young children.

It is known that nucleotides play various important roles in cellular metabolism. As precursors of nucleosine-phosphates (e.g., AMP, ADP and ATP) they have a metabolic function in signal transduction pathways (cyclic AMP and cGMP). An interesting phenomenon is that enterocytes can exhibit *de novo* synthesis of nucleotides as well as direct uptake. This may be of critical importance to the growing gut.

ORAL TOLERANCE IN RELATION TO AUTOIMMUNE DISEASE

Oral tolerance or immunoparalysis was defined as suppression of a systemic immune response upon oral administration of the antigen involved. In mammals, adults can be tolerised only by administering high doses, while neonatals require only low doses that should be administered continuously. It is long since known that systemic injection of albumin results in a stronger immunoparalysis when it is preceded by its oral administration. This holds generally for nominal (non-proliferating) antigens but is also the case for lysates of bacteria from the individual's indigenous flora. For some bacteria of the indigenous flora however, there is no tolerance. Such bacteria exert a systemic immune response and may play a role in autoimmune diseases such as IBD. One mechanism for IBD could be that in the case of mucosal damage and contact between non-tolerised bacteria in the gut flora and the systemic immune system, the reaction in the lamina propria leads to inflammation and consecutive damage of the lining. In this way a vicious circle develops. Autoimmune diseases of remote organs (such as rheumatoid arthritis) were also discussed in relation to (somewhat disappointing) experiments on oral administration of collagen fibres in comparison to control group. Beta-2 glycoprotein, produced in liver, is present at high concentrations in neonatals and may play an important role in the induction of tolerance to foreign antigens.

Vaccination may also have an effect on the establishment of autoimmune diseases. The relationship between hepatitis B virus vaccination and multiple sclerosis was mentioned, indicating that vaccination may not always be safe.

It was concluded that allergies develop via a different pathway than au-

toimmune diseases. The antibody isotypes involved are obviously different (IgE vs. IgG, respectively). The spectrum of tolerance developed by new-borns in Estonia and Pakistan may be different from that of new-borns in Sweden and may largely be due to the difference in contact with micro-organisms in the environment, i.e., by hygienic circumstances. This may explain the difference in allergies encountered in children raised in the Western world and those raised in developing countries.

IDIOTYPIC NETWORKS: THE INSTRUCTION OF THE IMMUNE SYSTEM DURING THE PERINATAL PERIOD

The meeting avoids the use of the word "idiotype" but accepts that there are antibodies that are directed towards other antibodies. Ab1 is the idiotype; Ab2 is the anti-idiotype.

The idiotypic network, part of the innate defence system, was conserved from ancient host defence pathways from our evolutionary forefathers. The system can be modulated by the thymus-dependent humoral immune system of the mother during pregnancy and during the lactation period. The transplacental and lactational transfer of antibodies from the mother to the new-born forms an important modulator of the foetal immune system and prepares the new-born for its encounter with environmental antigens.

The composition of the gut flora of a normal euthymic mother and the interaction with her T-cell system before and during pregnancy determines the degree of chronic graft vs. host disease (GvHD) following bone marrow transplantation experiments in her offspring (see Heidt, Veenendaal and van der Waaij elsewhere in this volume). Whereas acute GvHD is primarily T-cell mediated, late onset or chronic GvHD is predominantly antibody mediated.

B cell deficiency implies the possibilities of a hole in the antibody repertoire. Such a deficiency can be induced by the administration of an anti-idiotypic antibody to a mouse in a defined period shortly after birth. This throws a hole in the repertoire in the sense that the mouse is sensitive to a challenge of a bacterium for which the idiotypic antibody had affinity. The workings of the idiotypic antibody is thus affected by an anti-idiotypic antibody and there is an obvious consequence for later in life.

This has consequences for bone marrow transplantation experiments. During the period shortly after birth a hole in the antibody repertoire develops depending on the interaction of the immune system of the mother and her own microflora. This determines her IgG spectrum, and - since IgG's cross the placental barrier - also determines the polyspecific antibody repertoire in her young. It seems from literature that the acquisition of a restricted repertoire of antibodies is important during a certain phase early in life in order to have a broader repertoire later on in life and can also be used to protect offspring via immunisation of the mother during pregnancy.

COMMON THERAPEUTIC APPROACHES: INFLAMMATION AND CANCER

If bone marrow transplantation without GvHD would be possible, then this treatment could be used to cure autoimmune diseases like multiple sclerosis.

Currently, the bone marrow transplantation cannot guarantee success as GvHD lesions may be as severe as the autoimmune disease itself. More research on the interaction between the microflora and the immune system of bone marrow donors is urgently needed, specifically in relation to the effect of the graft on the recipient and its microflora.