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The Role of Mucosal Microbiota in the Development, Maintenance, and Pathologies of the Mucosal Immune System

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DEVELOPMENT OF THE SYSTEMIC IMMUNE SYSTEM: ROLE OF ENVIRONMENTAL ANTIGENS

A comprehensive review of the history and role of intestinal microflora is given in the version of this chapter presented in the previous edition of this volume (Cebra et al., 1999). The pioneering studies concerned whether the presence of bacteria in the intestine was essential for the life of the host (see Leidy, 1849). The general approaches involved either deriving mammals with a sterile intestinal tract (axenic or “germ-free” [GF]), which could then be deliberately colonized with known members of the intestinal microbiota (gnotobiotic mammals), or analyzing newborns, born essentially GF, as they developed and naturally acquired a “normal” microbiota. Early on, changes in the systemic immune system were monitored, since the somewhat separate mucosal immune system had not yet been properly

defined and appreciated. The early pioneers in GF/gnotobiotic studies of mammals included Glimstedt (1932) and Gustafsson (1948) in Sweden, Reyniers (1932), Pleasants, Wostmann, and Pollard at the Lobund Institute, in the United States (see Carter and Pollard, 1971); Miyakawa *et al.* (1958) in Japan; and Sterzl, Mandel *et al.* (1960) in Czechoslovakia. Pleasants, Wostmann, and their coworkers further minimized microbial and food antigen stimulation of mice by developing “antigen-free” (AF) mice fed a chemically defined (CD) diet of water-soluble, low-molecular-weight nutrients plus soy oil containing vitamins (Pleasants *et al.*, 1986). From earlier times (van der Waaij, 1969) up to the present (Fagarasan *et al.*, 2002), investigators have sought to simplify the elimination of gut microbes and circumvent the intricate, complex, and expensive procedures used to derive and maintain GF or AF rodents by decontaminating them with “cocktails” of orally administered antibiotics. Usually, the “physiologically normal”

state of hypertrophy and inflammation of the lymphoid system is somewhat reversed but there is little information of the effects on the developmental status and functional activity of particular elements of the immune system. However, the many analyses of the effects of intestinal colonization with microbes of GF or AF mice and of natural colonization of neonates on the systemic immune system can be generalized as follows (see Cebra *et al.*, 1999, for a more detailed account).

Conventionally reared (CNV) neonates develop “natural” plaque-forming cells (PFC) or antibody-secreting cells (ASC) in spleen and peripheral lymph nodes (PLN) against autoantigens, bacterial antigens (Ags) (especially lipopolysaccharide [LPS]), and sheep red blood cells (SRBC), whereas GF neonates are greatly retarded in this development (Tlaskalova *et al.*, 1970, 1983; Tlaskalova and Stepankova, 1980).

GF and AF adults can and do respond to active parenteral immunization with SRBC, protein Ags, and hapten-protein conjugates and to colonization by gut bacteria with splenic PFC or ASC, often at higher frequencies or titers than their CNV counterparts (Berg and Savage, 1975; Kim, 1979; Bos and Ploplis, 1994).

GF neonates, especially colostrum-deprived piglets, show a profound hypotrophy of PLN and spleen and a marked diminution of peripheral lymphoid cells reactive versus mitogens or polyclonal stimuli (Tlaskalova *et al.*, 1970; Tlaskalova and Stepankova, 1980; Kim, 1979). The hypotrophy persists into adulthood for both GF and especially AF mice, although the latter do show nearly normal numbers of splenic marginal zone and peritoneal cavity (PeC) B cells relative to their CNV counterparts (Bos *et al.*, 2003). The GF neonates also respond poorly to deliberate Ag stimulation (Tlaskalova and Stepankova, 1980; Kim, 1979; Tlaskalova *et al.*, 1994). The neonatal hyporesponsiveness of GF and even CNV mammals, especially versus microbial polysaccharide Ags, has often been attributed to immaturity of B cells and the time lag in transition of recently arrived B cells in the periphery (“virgin B cells”) to B cells positively responsive to cross-linking of BCRs (primary B cells). It has long been proposed that gut microbial stimulation, especially via LPS, can drive this process. Recently, Monroe and his students have shown a delay in the development of competence of neonatal B cells (until 18–23 days of life), to respond to *in vitro* cross-linking of their BCRs with F(ab')₂ anti-mouse IgM (Monroe *et al.*, 1993; Yellin-Shaw and Monroe, 1992). Neonatal and adult peripheral B cells are about equally responsive to mitogenic stimulation with LPS. Finally, this group further found that addition of LPS to cultures of neonatal B cells unresponsive to the presence of F(ab')₂ anti-IgM markedly enhanced their reactivity (Wechsler-Reya and Monroe, 1996). Thus, microbial products such as LPS may naturally enhance specific B cell responsiveness to cross-linking of their BCRs *in vivo* during neonatal development via microbial colonization of the gut.

DEVELOPMENT OF THE MUCOSAL IMMUNE SYSTEM: ROLE OF MICROFLORA

Most mucosal sites of lymphoid tissue—respiratory tract, adenoids, salivary glands, urogenital tract—in healthy mammals are in a quiescent state and generally resemble the status of lymphoid areas in spleen and most PLN. The intestinal tract, palatine tonsils, and occasionally the nasal-associated lymphoid tissue (NALT) are the exceptions. These mucosal lymphoid tissues are in a “physiologically normal state of inflammation” (Weinstein *et al.*, 1991; Liu *et al.*, 1991). Indeed, Prof. Hall (1984) has pointed out that were the respiratory tract-associated lymphoid tissues (RALT) in the same state as the gut-associated lymphoid tissue (GALT), we would all suffer from chronic pneumonia and bronchitis. It is the thesis of this chapter that intestinal microbes drive the development of GALT during neonatal life and act to maintain its physiologically normal steady state of inflammation. Specific and adaptive, “natural” and semispecific, and aspecific elements of the mucosal immune systems may benefit and be activated by host interactions with environmental Ags. To provide the experimental rationale for implicating intestinal or oral/nasal microflora in the development of GALT, palatine tonsil, and sometimes NALT and their steady state of inflammation, we must briefly contrast the status of systemic lymphoid tissue in healthy mammals—spleen, PLN—with GALT, palatine tonsils, and NALT.

GALT compartments

Early on, the term *GALT* was meant to include only the “inductive” sites in gut-associated lymphoid tissues, for lymphoid cell activation and proliferation (Brandtzaeg and Farstad, 1999), following the earlier usage of BALT (bronchus-associated lymphoid tissues). Recently, the inclusion of appreciable numbers of lymphoid cells from solitary follicles in typically isolated single-cell suspensions from gut lamina propria (LP) has been appreciated, blurring the distinctions between “inductive” and “effector” sites (see Hiroi *et al.*, 2000). Perhaps, future directed immunohistochemical analyses, microdissection (see Hamada *et al.*, 2002), and analyses using gene arrays on microchips after laser capture of cells will permit clear distinctions between cells in solitary follicles and those in the loose connective tissue of the gut LP itself. Thus, we feel that the inclusion of gut LP as one of the divisions of GALT is presently appropriate. The recognition of “atypical” subsets of T cells, natural killer (NK) cells, and NK-like cells in the intraepithelial spaces and the rather broad or “aspecific” reactivity of some of them with microbial products make their local activation likely and their inclusion as a compartment of GALT likewise appropriate.

Peyer's patches (PP) and solitary follicles (SF)

Peyer's patches (about 8–10) and SF are found in the walls of the small intestine or both small and large intestine,

respectively. About 30,000 SF occur in the human intestine, and about 10–20 SF occur in the murine small intestine, as just recently described (Brandtzaeg and Farstad, 1999; Hamada *et al.*, 2002). These organized lymphoid structures are composed conspicuously or mainly of spherical B lymphoid follicles, including chronically present B lymphoblasts embedded in a meshwork of follicular dendritic cells (DC). Both GALT and palatine tonsillar B cell follicles are secondary and display chronic germinal center reactions (GCR) (Weinstein *et al.*, 1991; Liu *et al.*, 1991). The predominant non-IgM isotype expressed on GC B blasts in the PP and SF of most mammalian species is IgA; B blasts in human tonsillar GC mainly express IgG isotypes (Lebman *et al.*, 1987; Pascuel *et al.*, 1994). Both these tissues normally contain relatively large numbers of IgA (Peyer's patches) or IgG (tonsils) "memory" B cells. Thus, the B cell follicles of these mucosal tissues differ from those of spleen and lymph node, which are ordinarily quiescent and "primary," displaying no GCR with dividing B blasts and being composed of IgM/IgD-positive primary B cells. The B cell follicles of PP, SF, and also murine NALT contain microfold (M) cells, scattered through their follicle-associated epithelium (FAE). These M cells act as "efferent lymphatics," transporting samples of foreign antigen from the gut lumen or airways into the organized lymphoid tissue (Weltzin *et al.*, 1989; Hamada *et al.*, 2002; Zuercher and Cebra, 2002; Zuercher *et al.*, 2002). Although the SF of mice share many characteristics of PP and differ from "cryptopatches" (see Kanamori *et al.*, 1996), they appear to also contain some of the cells characteristic of cryptopatches, especially the *c-kit*⁺, *IL-7R*⁺, *Thy1*⁺ cells involved in lymphopoiesis of some gut T cells (Hamada *et al.*, 2002).

Most of the T lymphocytes in PP reside in the wedge-shaped, interfollicular regions and include both *CD4*⁺ and *CD8*⁺ T cells (London *et al.*, 1990). Both of these subsets generally include a higher proportion of cells displaying activated phenotypes, such as *CD45RB*^{low}, *CD69*⁺, and *CD62L*^{low} (Talham *et al.*, 1999; Jump and Levine, 2002), than corresponding subsets from quiescent spleen or lymph nodes. Recently, *in vitro* culture of PP T cells in anti-*CD3*-coated plates has shown that a "naturally activated" population of *CD45RB*^{low}, *CD4*⁺ T cells can be stimulated via TCR to produce much greater amounts of *IL-10*, but not *IFN* γ , *IL-4*, or *IL-12*, in comparison with MLN and PLN (Jump and Levine, 2002). Thus, the PP seems to contain a set of T cells that express similar properties to those that may mediate oral tolerance or downregulation of peripheral T cells.

Interdigitating DC from PP of CNV mice (*CD11c*⁺ DC) have been shown to selectively induce production of *IL-4* and *IL-10* by naïve *CD4*⁺ T cells from TCR-transgenic mice (Iwasaki and Kelsall, 1999). Such DC are likely also found in gut LP and MLN (see the section on Gut Lamina Propria). Recently, Iwasaki and Kelsall (2001) have distinguished three subsets of DCs in PPs. The *CD11b*⁺/*CD8* α ⁻ (myeloid) DCs, localized to the subepithelial dome, appear

to be the subset that produces *IL-10* upon stimulation with *CD40*-ligand trimer and that can present Ag-peptide to stimulate *CD4*⁺ T cells to produce *IL-4* and *IL-10*.

Gut lamina propria

The gut LP is a meshwork of connective tissue underlying the gut epithelium and containing a broad spectrum of myeloid, lymphoid, and mesenchymal cells. With respect to B cell status, this gut compartment is roughly equivalent to medullary cords of lymph nodes. However, the B blasts and plasma cells in the LP of GALT in most mammalian species mostly secrete IgA antibodies (Crabbe *et al.*, 1965; Crandall *et al.*, 1967), whereas the splenic and lymph node plasmablasts largely express other immunoglobulin isotypes. Because of the recent recognition of SFs in the mouse small intestine and the usual manner of preparing dispersed cell suspensions with collagenase, dispase, and other enzymes, which includes the contents of the SFs in "LP preparations," the whereabouts and preferential localization of *B220*⁺, *IgM*⁺ B cells within this compartment is presently unclear. Certainly SF are rich in such cells (Hamada *et al.*, 2002).

The turnover or half-life of IgA plasmablasts in the gut LP was determined in neonatal mice (days 15 to 35 after birth) by chronic injection of ³H-thymidine at a time when this compartment was filling with these cells (Mattioli and Tomasi, 1973). A half-life of 4.7 days, under normal conditions of gut colonization of neonates with microbes, was found. However, given the influence of suckled maternal antibodies on forestalling gut colonization with some bacteria and the consequent delay in expressing the normal steady-state of IgA production in the gut until after weaning (days 22–24) (Jiang *et al.*, 2001), these periweaning estimations should be revisited in adult mice. We believe this reanalysis is especially important in view of the identification of long-lived plasma cells, functional in systemic antibody production (Slifka *et al.*, 1998; O'Connor *et al.*, 2002), and the long-term persistence of LP IgA-forming cells after the disappearance of PP GCR provoked by gut colonization with microbes (Shroff *et al.*, 1995) or the elimination of a gut-colonizing microbe by a shift from conventional to chemically defined diet (see below).

The *CD4*⁺ T cells of gut LP are generally more activated than splenic or lymph node counterparts, and they tend to express locally lymphokines associated with the Th2 subset of *CD4*⁺ T cells (Taguchi *et al.*, 1990). Recently, the construction of TCR transgenic mice expressing a receptor for ovalbumin peptide, responsive to class II molecule presentation, has provided a convincing example of the activation stimuli that are effective in the gut and are likely microbial products: the transgenic mice contain large numbers of dividing, activated *CD4*⁺ T cells in their gut LP that are expressing endogenous TCRs, i.e., their own TCRs, not the transgenic TCR (Saparov *et al.*, 1999). If these transgenic mice are crossed onto a *RAG-2* (*-/-*) background, such activated gut *CD4*⁺ T cells are not present.

Both T and B cells that express their effector function (memory T cells, IgA-committed B blasts, etc.) in the gut LP “home” to this tissue via HEV-like blood vessels (Jeurissen *et al.*, 1987). This “homing” is initially accidental via the recirculation in blood but leads to selective lodging in mucosal tissue based mainly on lymphocyte expression of $\alpha 4\beta 7$ integrin (the so-called homing receptor) and its ligand on vessel endothelial cells in mucosal tissue, mucosal vascular addressin (MAdCAM-1) (Nakache *et al.*, 1989; Williams and Butcher, 1997; Butcher *et al.*, 1999). At least IgA blasts then respond to the chemokine TECK (thymus-expressing chemokine), or CCL25 as it is presently named, a chemotactic factor made by gut epithelial cells. Thus, after “docking” via an $\alpha 4\beta 7$ -MAdCAM-dependent process, the IgA blasts are motivated directionally by a CCL25 gradient (Bowman *et al.*, 2002). A further factor in selective lodging of these cells in gut LP, leading to eventual local IgA production by IgM⁻, IgA⁺ or IgM⁺, IgA⁻ (already expressing I- α transcripts) B cells, is their responsiveness to IL-15 to promote IgA expression (Hiroi *et al.*, 2000). This particular responsiveness, which involves upregulation of IL-15R expression, is especially attributed to B-1 cells. The distinction between B-1 and B-2 cells in the gut LP is based on level of B220 (CD45R) expression. However, in the gut B220 expression may be more of a developmental marker rather than a B-subset marker. This matter may be resolved by distinguishing SF cells from bona fide LP cells. At any rate, IL-15 is also a product of gut epithelial cells (Reinecker *et al.*, 1996), like CCL25, and it remains to be determined whether gut microbial colonization induces the expression of either of these factors, since AF mice show negligible numbers of either B-1- or B-2-derived IgA plasmablasts in their gut LP (Bos *et al.*, 2003). A final likely candidate for facilitating the accumulation of IgA blasts in gut LP, pIgR, seems not to fulfill this function, as shown in pIgR (-/-) mice (Uren *et al.*, 2003).

Finally, the gut LP appears to contain DC that can be exposed to gut luminal Ags and act as antigen-presenting cells (APC) locally or emigrate to draining MLN, where they may prime T cells against gut-derived Ags (Liu and MacPherson, 1993). Such DC, recovered from CNV mouse MLN, can selectively stimulate naïve CD4⁺ T cells from TCR-transgenic mice to produce IL-4, IL-10, and TGF β (Alpan *et al.*, 2001; Akbari *et al.*, 2001).

Intraepithelial leukocyte (IEL) spaces

The intraepithelial leukocyte (IEL) spaces, found between absorptive epithelial cells and above basement membrane, are populated by a variety of small round cells, especially NK cells, NK-like T (NK-T) cells, and many CD8⁺ T cell subsets (Guy-Grand *et al.*, 1991; Goodman and Lefrancois, 1989; Bannai *et al.*, 2001). IELs are supposed to lodge between epithelial cells (EC) via their surface $\alpha E\beta 7$ integrin, reactive with the E-cadherin on the EC (Cepek *et al.*, 1994). Of particular interest as mediators of innate immunity are NK and NK-T cells. The former NK cells from IEL spaces are in a more activated state (based on killer-target ratios to achieve a given level of cytotoxicity) and target cell range than are NK cells from spleen or lymph nodes. It is interesting that the NK cells of the IEL spaces of CNV severe combined immunodeficient (*scid*) mice are more numerous and in a higher state of activation than those corresponding cells from CNV immunocompetent (*imcomp*) mice (these observations are shown in **Figs. 18.1** and **18.2**). The CD8⁺ T cells of the IEL spaces, unlike those from spleen and lymph nodes, display a much higher level of “constitutive” cytotoxicity without deliberate *in vitro* or *in vivo* activation (Lefrancois and Goodman, 1989). These findings are based on an assay, redirected cytotoxicity, which depends on coupling of putative cytotoxic T cells with target cells via antibody versus T-cell receptor (TCR) that also reacts with Fc γ receptors on target cells (Leo *et al.*, 1986). The abundance of “activated” NK

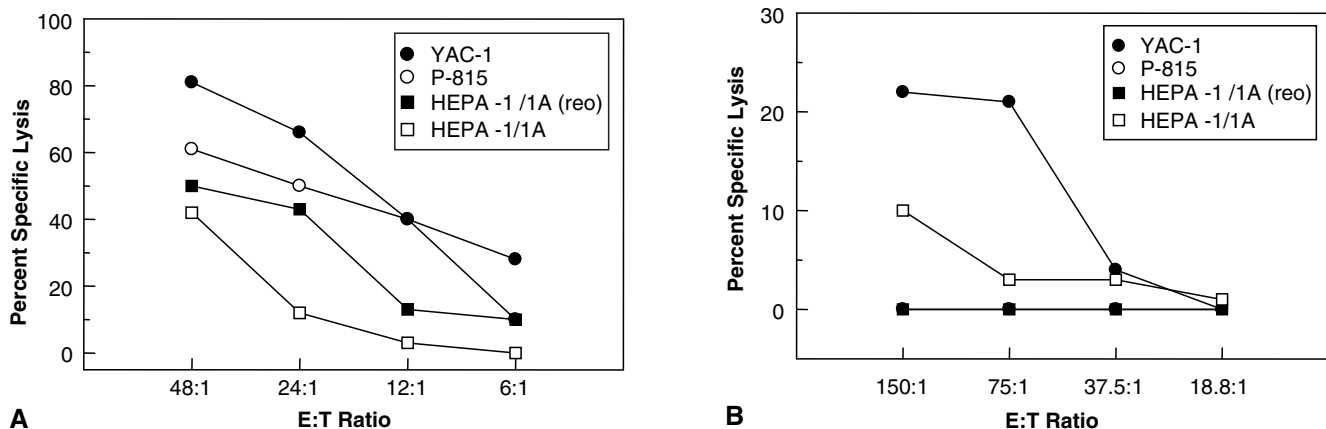


Fig. 18.1. Target cell range of IEL and spleen cells. IEL (**A**), and spleen cells (**B**), were isolated from C.B17 *scid* and assayed against YAC-1 (closed circles), P-815 (open circles), reovirus-infected HEPA-1/1A (closed squares), and noninfected HEPA-1/1A (open squares) in 6-hour ⁵¹Cr release assay at various E:T ratios. Spleen cells did not kill either virus-infected HEPA-1/1A or P-815 cells. Results shown are representative of five separate similar experiments (Cuff, C., and Cebra, J., unpublished data).

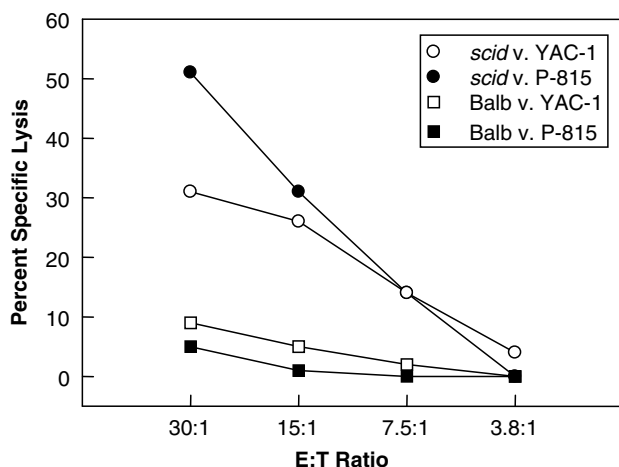


Fig. 18.2. Cytotoxic activity of IEL from CB.17 *scid* and BALB/c mice. IEL were isolated from groups of uninfected C.B17 *scid* (circles) and BALB/c mice (squares) and assayed against YAC-1 cells (open symbols) or P-815 cells (closed symbols) in 6-hour ^{51}Cr release assay at various E:T ratios. Typical results from one of four separate experiments are shown (Cuff, C., and Cebra, J., unpublished data).

and CD8⁺ T cells in the gut relative to those in spleen or lymph nodes suggests a chronic stimulation, likely by food and microbial Ags, and this matter will be considered below.

There is also an abundance of both conventional and unconventional NK-T cells in the IEL spaces of the large intestine (Bannai *et al.*, 2001; Heller *et al.*, 2002). These NK-T cells are either CD4⁺/CD8⁻ or CD4⁻/CD8⁻/ $\alpha\beta$ TCR⁺ and express NK1.1. Some of these express the invariant chain V α 14, J α 281, are restricted by the CD1 element (Bleicher *et al.*, 1990), and are responsive to α -galactosyl ceramide-type ligands (conventional) (see Heller *et al.*, 2002). Others of this NK-T subset are neither restricted by CD1 nor classical MHC class I Ags and are much more diverse in expression of TCRs (Bannai *et al.*, 2001).

Finally, ever since it was shown that DCs can extend processes from the LP, through the basement membrane, into the IEL spaces (Maric *et al.*, 1996), their role in facilitating microbial translocation and as APCs to directly activate B cells (Wykes *et al.*, 1998) and influence mucosal T cell differentiation (Alpan *et al.*, 2001; Akbari *et al.*, 2001) has been the focus of intense analyses (see next section).

INTERACTIONS OF THE INTESTINAL MICROFLORA WITH THE HOST: MAMMALIAN MODELS

Joseph Leidy (1849) wrote that “from the opinion so frequently expressed that contagious diseases and some others might have their origin and reproductive character through the agency of cryptogamic spores . . . I was led to reflect upon the possibility of plants of this description existing in healthy animals, as a natural condition; or at least apparently so, as in

the case of *Entozoa*.” Leidy reasoned that the wet epithelial surfaces of the body could provide a rich culture medium for commensal microbes. Perhaps the first systematic analyses of these commensal microbes was provided by Schaedler, Dubos, and their coworkers (Schaedler *et al.*, 1965a, 1965b; Dubos *et al.*, 1965). They stated that “mice and other mammals normally harbor an extensive bacterial flora, not only in the large intestine, but also in the stomach and small intestine. Although this flora plays an essential role in the development and well-being of its host, its exact composition is not known” (Schaedler *et al.*, 1965a). Unfortunately, their final lament is still true, although there have been significant advances in classifying and quantitating gut bacteria without *in vitro* cultivation. The most widely used methods for such classification rely on 16S rRNA sequence analysis via cDNA (see Amann *et al.*, 1995), and the use of distinctive oligonucleotides based on these sequences, either labeled for detection or used in microarray methodology, have allowed quantitation of particular fecal bacteria (see Amann *et al.*, 1995; Harmsen *et al.*, 1999; Rigottier-Gois *et al.*, 2003; Wang *et al.*, 2002). Promising novel approaches for “cultivating the uncultured” bacteria are also being developed (Zengler *et al.*, 2002).

The three seminal papers of Schaedler, Dubos, and coworkers offered the first comprehensive characterization of a portion of the gut microflora (using both aerobic and anaerobic *in vitro* culture) and employing the very models we still depend on today to assess the interactions of gut microbes with the GALT—the natural colonization of neonates and the deliberate colonization of axenic (GF) mice with particular gut commensal bacteria. The pioneering studies identified or established that the gut microflora included facultative anaerobes and obligate anaerobes such as lactobacilli, enterococci, and enterobacilli (including lactose-slow-fermenting *Escherichia coli*) in the former group and *Bacteroides* species and *Clostridium* species in the latter; that colonies of mice differed in the composition of those bacteria that could be cultured *in vitro*; and that there was a normal succession of colonization of the small and large gut with first lactobacilli, then enterococci, and then bacteroides in the large gut. Gram-negative enterobacilli were usually a minor component in the presence of the “complete” gut flora. These investigators also noted that certain populations of enterobacilli and enterococci decreased precipitously after having reached a maximum level. They found similarities between naturally colonized neonates and deliberately colonized GF mice with respect to succession of colonization and eventual localization in the gastrointestinal tract of particular microbial forms. Although no systemic or mucosal host immune responses were studied, these investigators speculated that the eventual outcomes of colonization with particular members of the indigenous microbiota might also allow a division between *normal microbiota* and *autochthonous microbiota*. The latter were supposed to have coevolved in a way that precluded a host response against self. Presently, immunological evidence that substantiates the concept of autochthonous microbiota remains inconclusive; rather, it would appear that the gut mucosal response to some luminal

microbes does not particularly compromise their continued, successful colonization (van der Waaij *et al.*, 1994; Friman *et al.*, 1996). For instance, a high proportion of the normal bacterial commensals of the human gut are found to be coated with “natural” IgA secreted into the gut lumen.

These early studies have led to the development of mice with a defined intestinal flora—“altered Schaedler’s flora”—and these represent oligo-associated, gnotobiotic mice carrying eight benign commensal enteric bacteria (Dewhirst *et al.*, 1999). Further definition of the normal intestinal microbiota of mice has led to discovery of a major colonizer, segmented filamentous bacteria (SFB) (Davis and Savage, 1974). These SFB have been shown to be related to clostridia (Snel *et al.*, 1995), and variants have been found in rats, pigs, chickens, and trout (Snel *et al.*, 1995; Meyerholz *et al.*, 2002; Yamauchi and Snel, 2000; Urdaci *et al.*, 2001). So far, these obligate anaerobic SFB remain uncultivable. Another notable normal commensal, *Helicobacter muridarum*, has been described by Phillips and Lee (1983) as a major colonizer of the murine colonic crypts. Finally, analysis of 16S rRNA libraries of mouse intestinal microbiota has revealed a large new group of intestinal bacteria (Salzman *et al.*, 2002). Further treatment of the microbiota of mice (Schaedler and Orcutt, 1983) and of humans (Mackie *et al.*, 1999; Savage, 1999) is available in excellent reviews.

Most of our present understanding of the role of gut microbes in stimulating the development of the host’s gut mucosal immune system and maintaining its “normally inflamed” steady state has come from combining elements of the original studies by Schaedler, Dubos, and coworkers but adding to these approaches contemporary *in vivo* and *in vitro* assays for the status of all the elements of both the mucosal immune system and the systemic immune system. Thus we divide this section into consideration of each element of the innate and adaptive mucosal immune system as it is perturbed in neonates by natural or deliberate microbial colonization, or in GF mice, deliberately colonized with *known* members of the gut flora, to yield “gnotobiotic” hosts.

Humoral mucosal immune system (adaptive and “natural”)

Shortly after the gut LP of several mammalian species (humans, rabbits, rats, and mice) was found to contain an abundance of secretory plasma cells (Crabbe *et al.*, 1965; Crandall *et al.*, 1967; Pierce and Gowans, 1975; Cebra *et al.*, 1977), most of which made IgA, it was noted that both GF adult mice (Crabbe *et al.*, 1970) and neonatal mice (Parrott and MacDonald, 1990) have a paucity of such cells. More recently, antigen-minimized mice (referred to as antigen-free [AF]) fed a liquid diet of hydrolyzed nutrients were found to have even fewer IgA blasts in their gut LP (Bos *et al.*, 2003). Thus, the absence of gut microbes seemed to forestall the natural development of the abundant population of IgA plasma cells normally present in gut LP. In the case of mice, the stimulatory effects of gut microbial products appears to apply to both B-2- and B-1-cell-derived IgA plasmablasts that accumulate in mouse gut LP (Bos *et al.*, 2003).

As early as 1968, Crabbe *et al.* (1968) were able to show that colonization of formerly GF mice with normal intestinal flora could stimulate the development of IgA plasma cells to normal levels within 4 weeks; in 1970, they showed that oral administration of the protein antigen ferritin to GF mice led to the appearance of antigen-specific IgA plasma cells in gut LP (Crabbe *et al.*, 1969). Pollard and his coworkers, in 1970 to 1971, made the significant observations that Peyer’s patches of GF mice contained mainly “primary” (quiescent) B-lymphoid follicles but that some enteric bacteria could activate GC reactions, while others were less effective (Pollard and Sharon, 1970; Carter and Pollard, 1971). Pollard and coworkers, Foo and Lee (1972), and Berg and Savage (1975) all agreed that some enteric bacteria were more effective than others in stimulating the development of specific circulating antibodies. Thus, they tended to support the notion of autochthonous versus normal gut microbiota. Of relevance is the somewhat more recent finding that the specificities of IgA antibodies in human milk seem to reflect the gut microbes that were present in a mother’s gut during the third trimester (Carlsson and Hanson, 1994). Many of the mammary IgA plasma cells seem to have been initially primed in GALT.

Coincident with these observations, in 1971 PPs were found to be sites for the preferred generation and accumulation of precursors for IgA plasma cells (Craig and Cebra, 1971), which could emigrate to and selectively populate all mucosal tissues (Cebra *et al.*, 1977). We now recognize that SF in both the small and large intestine may play a role similar to that of PPs (Brandtzaeg and Farstad, 1999; Hamada *et al.*, 2002). Thus, it became relevant to link the development of specific, IgA-committed B cells in PP and SF to the appearance and accumulation of specific IgA plasmablasts in the gut LP or elsewhere in mucosal tissues and to try to implicate particular gut microbes as effective stimuli of these perturbations.

Thus far we have established a set of general principles concerning gut microbial stimulation of the humoral mucosal immune system, while leaving some unresolved issues, as outlined in the next section.

IgA plasma cells and effective stimulators among the gut microbiota

Some commensal gut bacteria of the mouse stimulate the appearance of IgA plasma cells in gut LP of formerly GF mice better than others, and certain mixtures lead to gnotobiotic mice with almost two-thirds of the IgA plasmablasts that are found in CNV mice. Although no specific IgA responses in the gut or IgA plasmablasts in gut LP were addressed, *Bacteroides* and *Escherichia* species were found to be more effective than a variety of other microbes, including lactobacilli, enterococci, clostridia, corynebacteria, and actinobacilli. A mixture of four different gut microbes, used to colonize GF mice, came closest to simulating the development of a steady state of IgA plasma cells, comparable to that in the LP of CNV mice over a 4-week period (Moreau *et al.*, 1978).

These observations raise the following two questions. Are particular members of the normal gut microflora especially

effective at driving the development and maintaining the steady state of the humoral mucosal immune system in the gut? Do particular members of the normal gut microflora differ in stimulating specific versus polyclonal (aspecific) IgA production?

Although mixtures of gut microbial species, each successfully cultivated *in vitro*, have been shown to stimulate the development of IgA plasma cells in gut LP of formerly GF mice to roughly one-half to two-thirds of the levels from that in conventionally reared mice (Moreau *et al.*, 1978), most associations with single gut commensals are far less effective, quantitatively and temporally (Carter and Pollard, 1971; Foo and Lee, 1972; Berg and Savage, 1975; Moreau *et al.*, 1978; **Table 18.1**). In the last decade, we have appreciated that major elements of the gut flora of mice and of many other animals were obligate anaerobes that have not yet been cultured *in vitro* (Tannock *et al.*, 1987). Indeed, Joseph Leidy (1849) described a dominant gut morphotype, segmented filamentous bacteria (SFB), which he found in the midgut of termites. This SFB was tentatively named *Arthromitis* (jointed thread), and similar morphotypes have been found in the chicken, rat, mouse, pig, and trout (Snel *et al.*, 1995; Meyerholz *et al.*, 2003; Yamauchi and Snel, 2000; Urdaci *et al.*, 2001). The vertebrate

versions of this morphotype are gram-positive, segmented, obligate anaerobes that are spore formers (Davis and Savage, 1974). Savage and coworkers recognized SFB as a major component of the gut microbiota of mice and were able to enrich for these noncultivable bacteria by isolating intestinal epithelial cells, to which the 5- to 20- μ m SFB attached firmly via a holdfast segment that interdigitated with but did not penetrate the brush border (Davis and Savage, 1974; Tannock *et al.*, 1987). The SFB of mice has recently been isolated by the Nijmegen group, using treatment of fecal material with organic solvents to kill vegetative organisms, followed by limiting dilution to colonize formerly GF mice (Klaasen *et al.*, 1991). The latter limiting dilution was made possible by the finding that the vast majority of spore-forming, obligate anaerobes in postweanling to young adult mice were SFB. Snel *et al.* (1995) have used sequencing of 16S RNA to position the SFB of mice, rat, and chicken as a closely related group *within* the larger cluster of *Clostridium* species. Their relationship with Leidy's *Arthromitis* is still not clear.

Perhaps of greater relevance, the Nijmegen group has found that monoassociation of formerly GF mice with spores of this single gut commensal, SFB, provides a profound stimulus for the development of IgA ASC in the gut LP (Klaasen

Table 18.1. Natural IgA And Specific IgA Production in Germ-Free Mice Monoassociated with Individual Bacteria

Bacterium	Mouse Strain	Days after Colonization ^a	Total IgA (ng/ml) ^b	Specific IgA (ng/ml) ^c	% Specific IgA
<i>Listeria monocytogenes</i> actA(-) ^d	GF C3H	21	2200	322	14.6
<i>Morganella morganii</i>	GF C3H	28	924	44	4.8
Segmented filamentous	GF C3H	14	2460	33	1.3
<i>Ochrobactrum anthropi</i>	GF BALB/c	54	560	0	0
<i>Helicobacter muridarum</i>	GF BALB/c	14	491	4	0.8
<i>Escherichia coli</i> , Schaedler	GF BALB/c	30	462 (SI) 648 (PP)	118 (SI) 80 (J) 67 (I)	25.5 (D) 17.3 (J) 14.5 (I)
ASF	GF BALB/c	70	2250 (SI) 2150 (PP)	na	—

^aTime of maximal specific antibody output after colonization.
^bIgA production was determined in Peyer's patches (PP) and small intestinal (SI) fragment cultures by radioimmunoassay. Typical values for output of total IgA from PP and SI fragments cultures are: 3000–4000 ng/ml for CNV mice and 100–200 ng/ml for GF mice.
^cSpecific IgA production was determined by radioimmunoassay on plates that were coated with lysates derived from the involved bacteria.
^d*L. monocytogenes* act A(-) is a mutant strain of *Listeria* in which the actA gene is inactive. The actA gene is important in translocation of *Listeria* across epithelial cells. *M. morganii* is a gram-negative commensal bacterium that can translocate into the host but has not been shown to be pathogenic. Segmented filamentous bacterium is a strictly anaerobic commensal bacterium that cannot be grown outside of the host. *O. anthropi* is an aerobic gram-negative bacterial strain that grows poorly in the intestinal tract and almost does not translocate into the host. *H. muridarum* is a commensal bacterium that has been described as living in the crypts of the large intestine and has no history of pathogenic properties. *E. coli* Schaedler is gram-negative representative of commensal mouse flora with slow fermentative activities. ASF (altered Schaedler flora): eight microorganisms including *Lactobacillus acidophilus* (ASF 360), *Lactobacillus salivarius* (ASF 361), *Bacteroides distasonis* (ASF 519), *Flexistipes phylum* (spiral-shaped ASF 457), *Clostridium* cluster strains, and extremely oxygen-sensitive fusiform bacteria (ASF 356, ASF 492, ASF 502, and ASF 500). Abbreviations: na = not applicable; D = duodenum; J = jejunum; I = ileum (from Bos *et al.*, 2001; Boiko, N., and Cebra, J., unpublished).

et al., 1993). The magnitude of the response suggests that it is polyclonal, composed of much “natural” IgA; however, the rapid but delayed rise to preeminence of SFB attached to epithelial cells of the small intestine between 4 and 12 weeks of age, followed by exclusion of SFB to the cecum and large bowel (mostly unattached), suggests a specific component of the host’s response (Snel *et al.*, 1998). Indeed, we find that formerly GF *scid* mice, monoassociated with SFB, do not clear SFB from their small intestine for up to 1 year. We have collaborated with the Nijmegen group to evaluate SFB-specific versus polyclonal (“natural”) IgA plasmablast development driven by gut association of formerly GF mice with SFB and to quantitate how effective this stimulus was compared with “normal” expression of IgA by CNV mice with a “complete” microbiota (Snel *et al.*, 1998; Talham *et al.*, 1999). Our findings were that GC reactions occurred in Peyer’s patches by 14 to 21 days postinfection, and these gradually waned over about 100 days of colonization; “natural” IgA production by GALT fragment culture followed GC reactions in PP and reached levels of 70% of that found from GALT of CNV mice; and specific anti-SFB IgA antibodies did develop over 7 to 12 weeks, but these never exceeded 0.5% to 1.0% of the total “natural” IgA output. Thus, it appears that SFB may be a major stimulus for the development of the “natural” IgA system. We do not yet know how the host’s specific immune response to SFB effects its disappearance from the small gut; the bacteria do take on a coat of endogenous IgA (see following section). However, the level of “natural” IgA remains rather constant for up to 100 days of colonization as GC reactions subside. Thus, mechanisms for maintenance of IgA plasmablasts in gut LP should be investigated. One mechanism that may account for the maintenance of IgA plasmablasts for over 100 days is bacterial DNA-mediated survival. Unmethylated CpG dinucleotide motifs in bacterial DNA seem to rescue splenic B cells from apoptosis (Yi *et al.*, 1998; Yi *et al.*, 1999).

We have sought to extend the analyses by Moreau *et al.* (1978) to include seven innocuous enteric organisms as monoassociates of formerly GF mice as well as the eight commensal microbes in the altered Schaedler’s flora (ASF) (Dewhirst *et al.*, 1999). We compared their ability to stimulate the expression of “natural” IgA in the gut as well as specific antibodies reactive with sonicates of the organisms. Although there may be only a “thin line” between gut commensal and pathogen (Gilmore and Ferretti, 2003), we defined an enteric species as innocuous if it caused no detectable pathogenesis after monoassociating formerly GF *scid* mice and did not interfere with normal breeding. We used organ fragment cultures of various sections of gut and of PP to evaluate total and specific IgA production (Logan *et al.*, 1991; Weinstein and Cebra, 1991). Table 18.1 shows that each particular enteric microbe induced a particular level and ratio of “natural” and specific IgA production in the gut. Some organisms (*Morganella morganii*, *Listeria monocytogenes* mutant) resulted in a relatively high proportion of specific antibody while others did not (*H. muridarum*, SFB). In agreement with Moreau *et al.* (1978), a cocktail of gut

commensals (the eight members of ASF) stimulated nearly normal levels of total IgA production, as did the single microbial colonizer, SFB.

Germinal center reactions (GCR) in PP

B lymphoid follicles in PLN and spleen of healthy animals are normally in a “quiescent” state, being composed of mainly primary, sIgM⁺, nondividing B cells. So too are the B cell follicles of PPs in GF or AF mice (Weinstein and Cebra, 1991; Bos *et al.*, 2003). Gut colonization of GF or AF mice leads to the development of chronic GCR in the core of B cell follicles of PP, composed of rapidly dividing B cell blasts enmeshed in a three-dimensional web of follicular dendritic cells. GC of peripheral lymphoid organs are sites of isotype-switching and of the accumulation of point mutations in the V(D)J region of productive genes encoding Igs, leading to “affinity maturation” of the antibodies expressed by the B blasts (see Liu *et al.*, 1992). These GCR have been generally supposed to be dependent on CD4⁺ T cells (see Kelly *et al.* 1995), cognate T/B interactions, and cells of the B2 lineage in mice (Linton *et al.* 1992). Similar processes have been supposed to occur in the chronic GCR of PP, but there are few data concerning this matter. Milstein’s group has used transgenic mice expressing a transgene for a kappa chain that participates in binding 2-phenyl oxazolone (Gonzalez-Fernandez and Milstein, 1993; Gonzalez-Fernandez *et al.*, 1994). They find extensive point mutations in the V κ /J κ of this transgene of clonotypically related GC B cells from PP of unimmunized donors. However, these do not show the type of selection processes associated with affinity maturation among transgenes of peripheral B cells in secondarily immunized mice. They suppose the transgene in PP B cells acts as a “passenger” gene, accompanying B cells expressing endogenous V κ /J κ genes of antibody reactive with environmental Ag in the gut. Certainly, deliberate oral immunization with effective TD Ag, such as cholera toxin (Fuhrman and Cebra, 1981) and reovirus type 1 (Weinstein and Cebra, 1991), results in accumulation of IgA-committed, memory B cells and in GCR in PP, but the specific GC B blasts have not been examined for pattern of point mutations in V-genes that could result in affinity maturation.

Recently, we have examined unselected B cells from PP and gut LP differing or not in their expected membership in the B2 or B1 cell lineages taken from a variety of CNV mice (Stoel *et al.*, in press). Our findings were similar in all cases: the CDR3 spectrotypes of V α -genes showed pauciclinality; the individual V α /D_H/J_H genes expressed gave sequences with few point mutations and short N-additions with respect to documented GL genes; and a surprisingly high incidence of clonotypically related sequences was noted in some samples, which exhibited few but unrelated point mutations. These observations are different from those with use of unselected or specific B cells from spleen (Berek *et al.*, 1991; Stoel *et al.*, in press) and suggest that many PP and gut LP B cells derive from a process different from clonal selection, expansion, and secondary Ag selection in GCR.

Both B2 and B1 cells likely contribute to the process generating gut IgA blasts expressing near GL genes (Stoel *et al.*, in press). Recently, TI-2 type Ags such as $\alpha(1\rightarrow6)$ dextran and (4-hydroxy-3-nitrophenyl) acetyl-Ficoll have been found to stimulate GCR in spleen, albeit rather vestigial, and the specific B cells showed few point mutations and no evidence of their clustering in CDR (Wang *et al.*, 1994; Lentz and Manser, 2001; Toellner *et al.*, 2002). Presumably these small GCR were initiated by cells of the B2 lineage in the absence of cognate T-cell interaction. These observations of splenic GCR raise the possibility that the sequences of expressed V-genes in IgA blasts from the gut that we have found may be due to B2 cell-initiated, vestigial GCR in PP, occurring in the absence of cognate T/B interactions. Indeed, with use of influenza virus challenge of the respiratory tract in chimeric mice carrying B cells lacking class II molecule expression (Sangster *et al.*, in press) or of reovirus challenge of the gut of TCR (-/-) mice (Zuercher and Cebra, unpublished), specific antibody was produced of non-IgM isotypes by mucosal-associated lymphoid tissue in the absence of appreciable increase in “natural” Ig. In the case of the reovirus challenge of TCR (-/-) mice, vestigial GCR were observed in PP. A possible mechanism for such apparently T-independent responses is the “presentation” of multivalent Ags by interdigitating and/or follicular DC to B cells in conjunction with their secretion of B lymphocyte stimulator protein (BLyS) or “a proliferation inducing ligand” (APRIL) to stimulate modest expansion and isotype-switching (MacLennan and Vinuesa, 2002; Litinskiy *et al.*, 2002). Microbial polysaccharide Ag or “rafts” of Ag determinants on the surface of microbes might be especially functional stimulants of gut lymphoid tissues, especially since the ability of interdigitating DC extending through IEL spaces to transport bacteria from the gut lumen has been recognized (Rescigno *et al.*, 2001).

We also do not know whether continued presence of specific IgA plasmablasts in gut LP depends upon continued colonization by the particular microbe. We have found that the waxing and waning of GCR in PP is commonly observed upon monoassociation of formerly GF mice with many different commensal enteric microbes such as SFB (Talham *et al.*, 1999). To support the need for continuous restimulation of PP with novel microbial Ag in order to maintain the chronic GCR, we find that supercolonization of SFB monoassociated mice with *M. Morganii* after their GCR have waned results in a new cycle of GCR (Talham *et al.*, 1999).

Contribution of cells of the B2 versus B1 lineage to IgA production in the gut LP

This subject has been comprehensively reviewed in this volume (Chapter 33), but here we emphasize a few points relevant to gut microbial/B cell interactions and comment on the specificities of these interactions.

The contributions of B1 versus B2 cells to the composition of gut IgA plasmablasts and to IgA secreted into the gut lumen have been estimated at greater than 50% (Kroese *et al.*, 1989) to less than 5% (Thurnheer *et al.*, 2003). Both

studies used transfer of congenic, Ig-allotype distinct B1 cells from adult PeC into recipients that were CNV adults and immunocompromised (Kroese *et al.*, 1989; Bos *et al.*, 2000)—*scid*, X-irradiated—or were GF neonates, treated with anti-host IgM allotype to delay B cell development (Thurnheer *et al.*, 2003). The latter pups, upon maturation, were monoassociated with one of three common gut commensal microbes. We believe that the latter mouse model is likely closer to the normal physiologic state of CNV neonates when the balance of their IgA plasmablasts is established in the gut. However, the contributions of B2 versus B1 cells to IgA plasmablasts can clearly be compensatory, if one or the other B cell lineage is impaired (Snider *et al.*, 1999).

Although we believe that B1 cells can populate the gut with IgA plasmablasts and some of these make IgA that reacts with a particular pattern of microbial Ags (Bos *et al.*, 1996), we have so far found little substantial evidence that indicates that these cells arise by specific Ag selection and stimulation to divide and/or differentiate into secretory plasma cells. Recently, Macpherson *et al.* (2000) have shown that TCR (-/-) mice can exhibit an antimicrobial IgA response in the gut and that a complex radiation chimera seems to express a predominance of IgA plasmablasts with the allotype of the transferred, semipurified PeC B1 cells; no specific responses were measured in the latter case. Unfortunately, TI responses are not acceptable criteria for B1-derived, gut IgA responses, and the radiation chimera—comparing transferred PeC versus BM B cells—suffers from the same criticisms as the original model (Kroese *et al.* 1989. see Thurnheer *et al.*, 2003). Furthermore, the latter chimeric model (Macpherson *et al.*, 2000) does not address specific Ag stimulation of B1 cells.

Recently, we have found that upon transfer of extensively purified, CD4⁺ T cell-free B1 cells into CNV *scid* recipients, no IgA plasmablasts develop in gut LP over 8–10 weeks unless CD4⁺ T cells are cotransferred (Jiang *et al.*, 2004). This synergy requires colonization of the host with enteric microbes, and apparently both B1 and CD4⁺ T cells benefit from the colonization. Apparently the T cell effect is of the “bystander” type since monoclonal DO11.10 (ovalbumin-peptide specific) TCR transgenic, RAG-2 (-/-) T cells are effective at facilitating B1-mediated IgA production in the gut. This effect occurs only if the transgenic cells are activated in the donor or, by ovalbumin feeding, in the recipient, and is not successful in GF recipients. Although the latter observation suggests T cell activation via TCR, either by ovalbumin or microbial Ags, it does not indicate whether the requirement of B1 cells for microbial colonization is specific or not.

It has previously been shown that LPS, given orally or intraduodenally, can activate peripheral B1 cells to secrete antibody (Murakami *et al.*, 1994). This is presumably a polyclonal activation that could be acting via the B1 cell LPS-R (TLR-4/CD14) or indirectly via activation of gut Tr1-type, CD4⁺ T cells through their TLR-4 to produce IL-10, a B1 cell growth and differentiation factor (O’Garra *et al.*, 1992; Caramalho *et al.*, 2003). Finally, IL-15 seems to be involved

in accumulation of B1 lineage-derived IgA plasmablasts in the gut LP (Hiroi *et al.*, 2000). Since gut epithelial cells (EC) can make IL-15 (Fehninger and Caligiuri, 2001), it is possible that enteric gut commensals can upregulate this synthesis. At any rate, the specific Ag selection and stimulation of B1 cells remain problematic.

Suckled maternal antibodies

Suckled maternal antibodies can forestall the natural development of active mucosal immune responses in neonates against gut microbes. It has been known for some time that the gut LP of newborns shows a paucity of IgA plasmablasts (Parrott and MacDonald, 1990) and that specific antigen-reactive B cells in PP committed to IgA (IgA memory cells) against the microbial determinants phosphocholine, β 2 \rightarrow 1 fructosyl (inulin [In]), and β -galactosyl take weeks after birth to rise to adult frequencies (Cebra, 1999). We speculated that “it seems plausible that changes in gut flora accompanying weaning or a decline in passively acquired maternal antibodies to In or both could result in increased natural stimulation of In-reactive cells at 3 to 5 weeks” (Shahin and Cebra, 1981).

To address whether neonatal mice had an underdeveloped or competent humoral mucosal immune system, we infected 10-day-old pups with reovirus 1 by the oral/intragastric route (Kramer and Cebra, 1995a). Reovirus 1 is a potent stimulator of both cellular and humoral mucosal immune responses in adult mice and is normally absent from our specific-pathogen-free (SPF) mouse colonies (London *et al.*, 1987). Thus, dams in our colonies have no circulating or mucosal antibodies against reovirus. Given orally to either 10-day-old pups or adults, reovirus 1 causes a transient intestinal infection without clinical symptoms. We found that pups expressed prompt, specific IgA antibody responses in 3 to 5 days, using the method of PP and small intestine organ fragment culturing. These responses were accompanied by distinct GC reactions in PP, with many IgA blasts, at a time, 14 to 17 days after birth, when nonimmunized pups still had quiescent B-cell follicles in PP. We also found that immunized pups expressed about 20 times more “natural” IgA than virus-specific IgA, whereas noninfected control pups made neither specific nor “natural” IgA. Apparently, 14- to 17-day-old pups are able to develop “natural” IgA responses but ordinarily do not do so until 22 to 25 days of age, unless challenged with an infectious enteric virus not commonly associated with the mouse colonies (Kramer and Cebra, 1995a).

We sought to evaluate possible effects of the immune system of both birth and nurse mothers on development of the neonatal mucosal immune system, especially on the expression of “natural” IgA (Kramer and Cebra, 1995b). We reciprocally crossed *imcomp* mice with coisogenic *scid* mice. The homozygous *scid/scid* mouse cannot express any form of specific, adaptive immunity, whereas the heterozygous F_1 mice (*scid/+*) are fully competent. Thus, the only difference between the two groups of F_1 mice, born to parents exposed to the same SPF flora, is whether they were born of *scid* or

imcomp mothers. F_1 mice could also be swapped at birth, so that pups born of *scid* mothers could be nursed on *imcomp* dams and vice versa. Without using any *deliberate* gut mucosal stimulus, we made a number of principal findings:

- F_1 pups born to and nursed on *scid* dams show a precocious rise in “natural” IgA production in PP and small intestine on days 15 to 16 after birth, with concomitant GCR in PP, whereas such expression of IgA is delayed at least until days 22 to 25 in pups born to *imcomp* mothers;
- This difference between the two groups of F_1 pups is reflected by IgA-secreting cells from PP, MLN, and gut LP; for instance, pups born of *scid* mothers have about 200 IgA ASC/ 10^6 in PP and 2000 IgA ASC/ 10^6 in the LP, whereas at day 16, pups born of *imcomp* dams have negligible ASC;
- The F_1 pups born of *imcomp* mothers have high levels of suckled, maternal IgA in their stomach contents, whereas it is absent from pups born of *scid* mothers; and
- The bacteria isolated from the intestine of pups born and nursed on *imcomp* dams are “coated” with IgA at an early age, whereas bacteria from pups of *scid* dams are initially “uncoated” but gradually, beginning on day 16 after birth, acquire an IgA coating via active production of IgA (Kramer and Cebra, 1995b).

The implication of these findings is that suckled, maternal IgA antibodies coat gut commensal bacteria in the neonatal intestines, blocking active mucosal immunization against members of the normal gut flora until weaning. In the absence of the shielding effects of passively acquired, maternal antibodies, neonates born of *scid* mothers are fully capable of developing gut IgA responses, PP GCRs, and gut LP IgA ASC against environmental antigens.

To directly implicate passively acquired, suckled maternal immune elements in forestalling the development of active, humoral mucosal immunity in the neonatal gut, we deliberately immunized some of the *imcomp* dams orally with reovirus (Kramer and Cebra, 1995a; Periwal *et al.*, 1997). Offspring of immune, nonimmune but *imcomp*, and *scid* mothers were also swapped for nursing. We took advantage of the stimulatory effect of enteric reovirus not only for virus-specific IgA antibodies but also for the development of “natural” IgA. The findings were rather consistent: oral vaccination of *imcomp* nurse dams had the greatest effect in preventing or retarding both virus-specific and “natural” IgA responses by the nursing pups; oral immunization of birth mothers had only subtle suppressive effects if the pups were then suckled on *scid* or nonimmune *imcomp* dams. We have sought to immunize neonates born of mucosally immune mothers actively by the oral route (Periwal *et al.* 1997). The aim was to allow the pups to benefit from the potentially protective effects of passively acquired maternal antibodies while still being actively immunized via the mucosal route. We have found that “live” reovirus 1, a protective vaccine against oral challenge with reovirus type 3 (Cuff *et al.*, 1990), can be encapsulated with spermine/alginate compounds. The

small (about 5 μm) capsules can be given orally to avoid neutralization by coincidentally suckled maternal antibodies, and these stimulate both active systemic and mucosal immune responses in the pups (Periwal *et al.*, 1997). It has been known for some time that lactating human mothers contain antibodies in their milk reactive with their own gut microbes, especially those present during the outset of the last trimester of pregnancy, and these could protect their nursing neonates against potential enteric pathogens (Carlsson and Hanson, 1987). Using a mouse model involving *imcomp* or *scid* dams, we have found that their immunologic competence is a factor in forestalling the development of the neonatal mucosal immune system of their nursing pups and that it is also associated with a delay in colonization by certain gut commensals, such as SFB (Jiang *et al.*, 2001).

Cellular mucosal immune system (innate and adaptive)

The most conspicuous compartments of the cellular mucosal immune system of the gut include the IEL spaces, the PP, and the gut LP. There is considerably less information concerning the role of gut microflora in the development and activation of the elements of the cellular versus humoral immune system. Generally, the approaches have used comparisons between neonates and adults and of GF or AF adults, before and after deliberate colonization with commensal microbes.

IEL compartment cells

The cells in the IEL compartment change in number and proportions of phenotypically/functionally distinct subsets during normal development and after colonization of formerly GF adult mice with gut microbes.

NK cells in the IEL spaces

NK cells (large granular lymphocytes) comprise a significant proportion of IELs in CNV rodents (Tagliabue *et al.*, 1982). Generally, their target cell range and potency (E:T ratio) is greater than that of NK cells in spleen or peripheral lymph nodes (see Fig. 18.1 for CNV *scid* mice). Gut infection of *imcomp* mice with coronavirus (Carmen *et al.*, 1996) and of *scid* mice with reovirus (Table 18.2) results in activation of gut NK cells in the IEL spaces. The CNV *scid* mice actually have IEL NK cells that display an activation state much greater than that of CNV *imcomp* mice before deliberate infection, that is, show greater potency in cytotoxic assays using YAC-1 targets or P815 (Fig. 18.2). Unlike CNV *scid* mice, the cytotoxic potency of NK cells in the IEL compartment of CNV *imcomp* mice is low and similar to that in their GF counterparts (Fig. 18.3). Colonization of the GF *imcomp* mice (C3H/HeJ) with SFB results in a marked increase in NK potency over a 14- to 23-day period postinfection, as measured with YAC-1, P815, and P388 target cells (Lee, 1995; Fig. 18.3 and Fig. 18.4). The mouse *E. coli* (Schaedler's), when used to monoassociate GF *scid* mice, also is associated with a rise in pan-NK-positive cells in IEL spaces, compared with the cohort in AF *imcomp* mice, but they do not reach the level of those in CNV *scid* mice (Fig. 18.5). The functional poten-

Table 18.2. Effects of Reovirus Infection on the Cytotoxic Activity of IEL from *scid* Mice

Group ^a	Weeks Postinfection			
	1	2	3	4
Noninfected	13.3 ^b	17.2	10.5	20.0
Infected	5.5	16.6	66.6	<5.0 ^c

^aGroups of 4–6 *scid* mice received 10^7 plaque forming units of reovirus serotype 1/L by the oral route. One to 4 weeks after infection, groups of infected and noninfected mice were killed, and isolated IEL were assayed for cytotoxic activity against ⁵¹Cr-labeled YAC-1 targets in a 6-hour assay.
^bResults are expressed as LU/ 10^6 cells to achieve 30% specific lysis.
^cSignificant morbidity and mortality are observed at 4 weeks postinfection.

tial and number of NK cells in the IEL compartment of GF *scid* mice have not yet been reliably determined. Thus, it seems likely that normal gut microbes can be partly responsible for the activated state of NK cells in the IEL compartment, relative to those in spleen and peripheral lymph nodes.

Mechanisms for the interaction of microbial products with NK cells in the IEL spaces have not been well studied. However, some possibly relevant observations have been made with use of splenic NK cells assessed *in vitro* or *in vivo*. Wherry *et al.* (1991) found that a heat-killed *L. monocytogenes* vaccine, along with macrophage-produced TNF α and another unknown factor, could stimulate splenic NK cells *in vitro* to produce IFN γ . Likely, the “unknown factor” was IL-12 (Hunter, 1996; Chace *et al.*, 1997). Further, differentiation of bone marrow precursors of NK cells requires IL-15, but so does the survival and maintenance of transferred, mature splenic NK cells as detected later in spleen, blood, and liver (Cooper *et al.*, 2002). Perhaps, NK cells in IEL spaces likewise require IL-15, made by gut EC (Fehninger and Caligiuri, 2001) and possibly induced by microbial products, for their maintenance. Also, microbial products originating in the gut possibly can activate NK cells and their expression of IFN γ . Finally, it is not clear why *imcomp* mice have less NK potency contributed by IEL cells than *scid* mice. Possibly, *imcomp* mice have secretory antibodies that can more effectively exclude gut microbial products from the various sites of the gut where NK cells are found.

T cells in the IEL compartment

The major set of T cells in the IEL spaces is CD8⁺, although many subsets of these are often present (Goodman and Lefrancois, 1989; Guy-Grand *et al.*, 1991). The CD8⁺ T cells in neonatal mice and in GF and AF mice include a much higher proportion of cells expressing γ/δ TCR than is commonly found in IELs from CNV adult mice (Lefrancois and Goodman, 1989; Guy-Grand *et al.*, 1991; Hooper *et al.*,

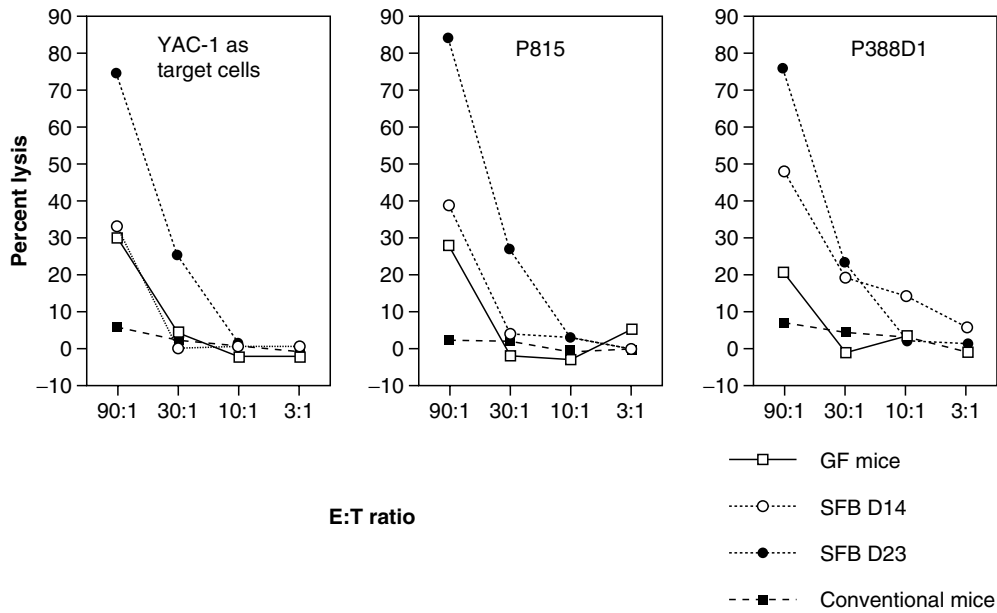


Fig. 18.3. Upon gut colonization of GF BALB/c mice with segmented filamentous bacteria, the cells of the IEL compartment show an increase in cytotoxicity versus NK- and NC-cell targets (YAC-1, P-815, and P-388 D1 cells) (Lee, F., and Cebra, J., unpublished data).

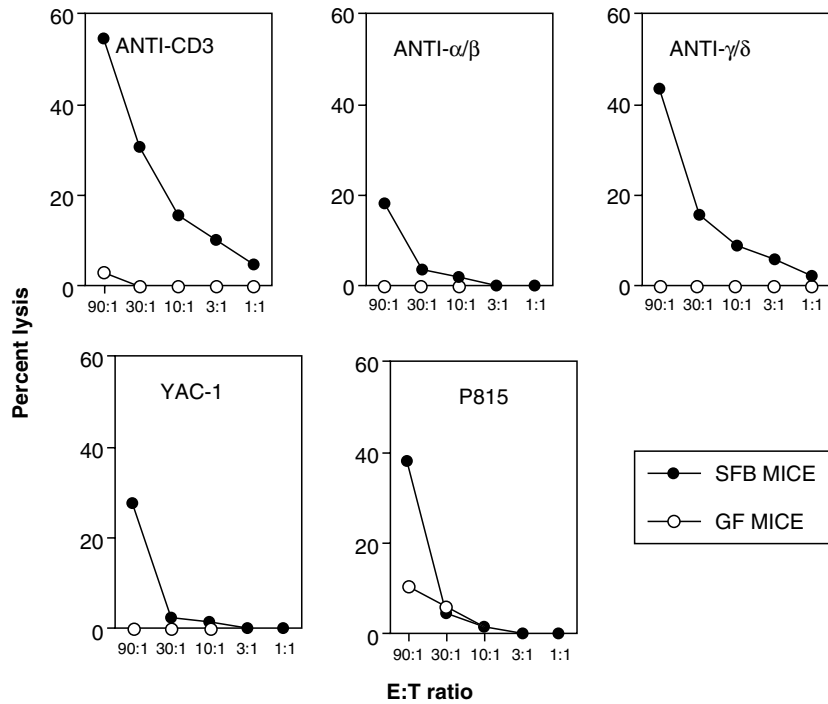


Fig. 18.4. Constitutively cytotoxic cells develop in the IEL compartment of formerly GF C3H/HeN mice following oral/gut colonization with SFB. IELs from GF mice were compared with those from formerly GF mice colonized for 60 days with SFB. Target cells were hybridoma lines making anti-CD3, anti-α/β TCR, or anti-γ/δ TCR (to detect various subsets of constitutively cytotoxic T-cells) and YAC-1 and P-815 (to detect NK and NC cells, respectively) (Lee, F., and Cebra, J., unpublished data).

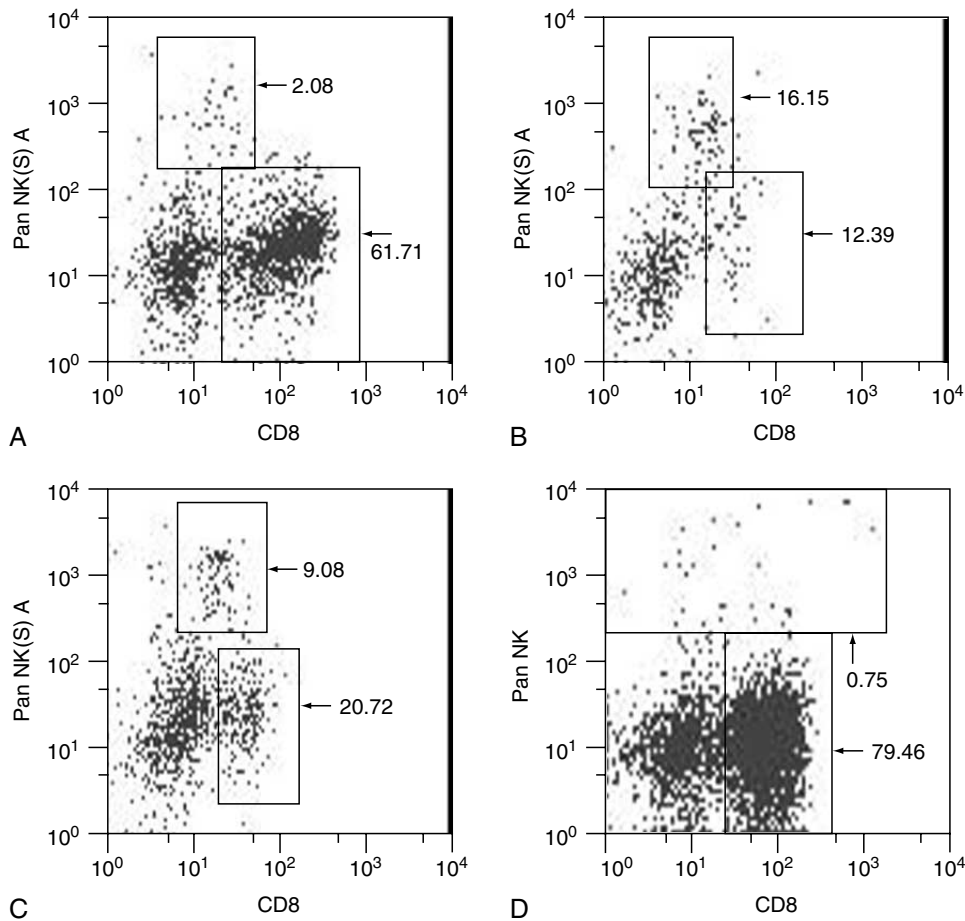


Fig. 18.5. FACS analyses of cells from IEL compartment of (A), AF *imcomp* BALB/c mice; (B), CNV *scid* mice; (C), GF *scid* mice after 24 days of monoassociation with Schaedler's *E. coli* (mouse commensal coliform bacterium); and (D), CNV *imcomp* BALB/c. (Boiko, N., and Cebra, J., unpublished data).

1995). Most of the CD8⁺ T cells in these latter rodents are Thy-1 negative and express the α/α rather than the α/β form of the CD8 molecule. The constitutive (redirected) cytotoxicity of these T cells is generally low (Lefrancois and Goodman, 1989). With maturation of mice, and especially after deliberate oral infection of adult GF mice with reovirus or gut commensal bacteria, the proportion of α/β TCR⁺, Thy-1⁺, and α/β CD8⁺ T cells increases (Cuff *et al.*, 1992, 1993; Umesaki *et al.*, 1993; Kawaguchi *et al.*, 1993). This increasing subset, which accounts for a twofold to fivefold increase in cellularity in the IEL compartment, includes both antigen-specific and constitutive cytotoxic and precytotoxic T cells. GF mice have very low levels of CD8⁺ T cell-mediated constitutive cytotoxicity (Lefrancois and Goodman, 1989). However, colonization of either formerly GF BALB/c mice (Umesaki *et al.*, 1995) or C3H (Lee, 1995) mice with SFB results in the progressive increase of Thy-1⁺, α/β TCR⁺, CD8⁺ T cells in the IEL spaces, which express the α/β CD8 heterodimer. These newly arising T cells appear to contain the constitutive cytotoxicity that increases among the IELs (Fig. 18.4). It is not clear what specificities are expressed by these T cells, apparently stimulated by SFB colonization.

Although reovirus-specific, CD8⁺ cytotoxic T cells seem to be generated in PP following viral entry via M cells and these then appear to migrate to IEL spaces (Cuff *et al.*, 1993), it is not clear how gut commensal bacterial colonizers mediate the accumulation of constitutively cytotoxic, α/β TCR⁺, α/β CD8⁺ T cells in IEL spaces. Perhaps gut interdigitating DC that extend their processes through IEL spaces to penetrate the gut EC monolayer can “sample” gut bacterial colonizers and their microbial products (Rescigno *et al.*, 2001). At any rate, following gut colonization of GF mice with members of their normal microbiota, continuous BrdU-labeling shows a preferential increase and labeling of these cells or their immediate precursors (Imaoka *et al.*, 1996). It is still not clear whether these CD8⁺ T cells in the IEL compartment respond specifically or polyclonally to microbial Ags and/or products such as TLR ligands.

Although the deliberate colonization of GF mice with commensal gut bacteria or infection with reovirus increases the α/β CD8⁺, α/β TCR⁺ IEL cells twofold to fivefold, molecular analyses of the V β genes expressed by this subset of IEL cells reveals that the population is oligoclonal but idiosyncratic for any given mouse (Regnault *et al.*, 1994). A comparison

of this subset of IEL T cells from CNV versus GF mice reveals the same degree of oligoclonality in each group, although the GF mice had 10-fold fewer of such cells (Regnault *et al.*, 1996). The generalization from this and other findings is that intestinal, microbial Ags are not a main selective stimulus for the development of this oligoclonality, and particular gut colonizers (SFB) may increase the number of these α/β TCR⁺, CD8⁺ T cells in the IEL spaces, but they do not appreciably alter the pattern of V β -gene expression when used to colonize formerly GF mice (Bousso *et al.*, 1999; Umesaki *et al.*, 1999).

Both the α/β and α/α CD8⁺, α/β TCR⁺ T cells in IEL spaces seem to require both IL-2 and IL-15 for their expansion and survival (Gelfanov *et al.*, 1995; Lodoice *et al.*, 1998; Lai *et al.*, 1999). The α/β CD8⁺, α/β TCR⁺ T cells seem to be a source for the local IL-2, while gut EC likely provide the IL-15 (Reinecker *et al.*, 1996). Only the α/β CD8⁺, α/β TCR⁺ T cells can mediate perforin-based cytotoxicity, while both they and α/α CD8⁺, α/β TCR⁺ T cells can kill via a Fas/FasL-based mechanism (Gelfanov *et al.*, 1996). However, we still do not know how benign bacterial colonizers stimulate the accumulation, survival, and activation of these T cells in the IEL spaces. Perhaps part of this process involves the upregulation of IL-15 production by gut EC.

The γ/δ TCR⁺ T cells appear to develop and then accumulate in the IEL spaces independent of gut microbial colonization (Bandeira *et al.*, 1990). Thus, this subset could be considered the T-cell counterpart of B1 cells, which develop and accumulate in the peritoneal and pleural cavities, although not in the gut LP (Bos *et al.*, 2003), independently of exogenous environmental antigens (Guy-Grand *et al.*, 1991; Hardy and Hayakawa, 1992). Although the γ/δ TCR⁺ T cells in the IEL spaces are prone to activation-induced death upon stimulation via their TCR, these cells also show enhanced survival promoted by exogenous IL-15 (Chu *et al.*, 1999). Perhaps microbial products can contribute to their activation, if not accumulation in IEL spaces, and also to their continued survival via EC-produced IL-15.

Responsiveness of CD4⁺ T cells in PP and gut LP

Gut microbial antigens may stimulate and activate CD4⁺ T cells in the PP and the gut LP. This topic is particularly relevant to the next section of this chapter, chronic noninfectious diseases, and will be addressed further there. Not many convincing studies have been published concerning the development of *specific* responsiveness of GALT CD4⁺ T cells to commensal microbial antigens. The difficulties include the polyclonal and/or mitogenic stimulants that accompany bacterial extracts and sonicates (MacDonald, 1995). However, the few studies in GF or AF rodents and their colonized gnotobiotic counterparts indicate the following. First, AF mice, deprived of exogenous stimulation, develop a normal repertoire of functional T cells (Vos *et al.*, 1992). Second, CD4⁺ T cells from PP of CNV *imcomp* mice exhibit “spontaneous” proliferation (autologous mixed lymphocyte reactions, or AMLR) to endogenous or exogenous APC; either GF or AF CD4⁺ T cells or APCs in cocultures

with their APC or CD4⁺ T cell counterparts from CNV mice respectively show diminished AMLR. These observations suggest that the GALT ordinarily contains both APCs charged with microbial antigenic determinants and reactive CD4⁺ T cells (Hooper *et al.*, 1994, 1995). Third, generally, the *in vitro* CD4⁺ T cell reactivity to microbial antigens is not manifest *in vivo*, although in patients with inflammatory bowel disease (IBD) or in animal models exhibiting IBD lesions, there is evidence that GALT CD4⁺ T cells do react with antigens of the endogenous microbial flora (Duchmann *et al.*, 1999). Fourth, we have found that CD4⁺ T cells in PP of CNV mice have a majority of CD45RB^{low} cells, indicative of prior exposure to antigens (Talham *et al.*, 1999). Spleen and PLN of such mice have CD4⁺ T cells that are mostly CD45RB^{high}, indicative of naive or unprimed T cells. In GF mice, the majority of PP CD4⁺ T cells are CD45RB^{high}. However, colonization of these mice with SFB activates their PP CD4⁺ T cells and shifts this population in 4 to 8 weeks to a CD45RB^{low} majority (Talham *et al.*, 1999). This observation suggests a nonspecific role for commensal gut organisms in activating GALT CD4⁺ T cells.

The specificities expressed by gut CD4⁺ T cells, via their TCR, for gut commensal bacteria have long been enigmatic. Attempts to implicate gut commensal bacteria as provocateurs of inflammatory bowel disease (IBD) in various mouse models have involved comparisons among fecal/bacterial extracts, medium alone, or extracts of food pellets and PMA/ionomycin to try to demonstrate bacterial product-driven proliferation or lymphokine production (usually IFN- γ or IL-2) by gut LP CD4⁺ T cells recovered from mice developing IBD (Brimnes *et al.*, 2001; Cong *et al.*, 1998). Generally, these T cell responses seemed to be dependent on MHC class II molecule expression and could be inhibited *in vitro* with specific anti-class II antibody. Also, the IBD development failed to occur in otherwise susceptible class II gene “knockout” mice (Matsuda *et al.*, 2000). In further support of specific stimulation by the gut microbial Ag was the finding of oligoclonality, albeit single-mouse unique, among TCR expressed by CD4⁺ T cells from mice with IBD, in CDR3- β spectrotyping analysis (Matsuda *et al.*, 2000). Other observations that indirectly support TCR/bacterial Ag interaction in the activation of gut CD4⁺ T cells come from analyses of the DO11.10 transgenic mouse. This mouse is transgenic for TCR reactive with an ovalbumin peptide in the context of I-A^d. While DO11.10 transgenic mice with a RAG-2 (-/-) background had few and quiescent (naïve phenotype) gut CD4⁺ T cells, the transgenic mice lacking knockout of the RAG-2 gene had plentiful, activated T cells in their gut LP, expressing many nonclonotypic, endogenous TCR (Saparov *et al.*, 1999).

Another example of the interaction of gut microbial products with T cells, either directly or indirectly, is seen in the phenomenon of “homeostatic proliferation” in irradiated *imcomp* mice, CNV *scid* mice, or GF *scid* mice. **Fig. 18.6** shows that splenic fluorescent dye (CFSE)-labeled CD4⁺, CD8⁺, or C4 transgenic CD8⁺ T cells, transferred separately to the CNV recipients, exhibit rapid homeostatic proliferation

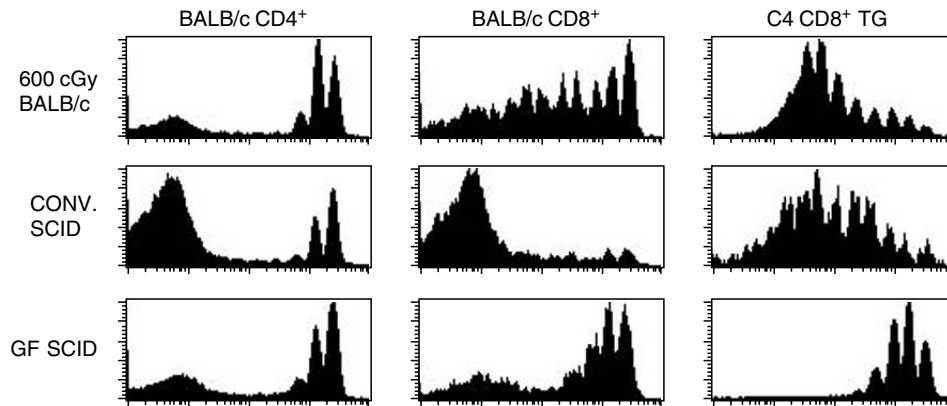


Fig. 18.6. An inoculum of 1×10^6 purified CD4⁺, CD8⁺, or C4 (monoclonal TCR) CD8⁺ T cells, fluorochrome-labeled with CFSE, was injected intravenously into irradiated *imcomp* BALB/c, *CNV scid* or *GF scid* mice. Four days later, splenocytes were analyzed by FACS for green fluorescence of the infected T cells. The abscissae show increasing intensity of fluorescence, from left to right (Suhr, C., Shen, H., Troy, A., Jiang, H.-Q., and Cebra, J., unpublished data).

over 4 days, while proliferation is minimal in *GF scid* recipients (Suhr, Shen, and Cebra, unpublished). Again, the specificities of TLR or TCR expressed by the responding T cells are unknown.

It has been difficult to demonstrate TCR-based specificity for a particular gut bacterium, even in monoassociated mice with IBD (Jiang, H.-Q., and Cebra, J. J., unpublished). Perhaps the findings of Caramalho *et al.* (2003) that CD4⁺ T cells may be activated by microbial products such as LPS, CpG, PG, and flagella via their TLR and that this stimulus can act synergistically with engagement of the TCR could support a rather broad specificity for T cells reactive with some microbial Ag. Examples of possibly broadly cross-reactive Ag expressed by bacteria include flagella, the N-formyl-methionyl peptides (Kerksiek *et al.*, 2001), and the S-polypeptides (Sleytr and Beveridge, 1999). It seems that a systematic comparison of individual gut microbial species bearing a variety of TLR ligands for reactivity with gut CD4⁺ T cells from monoassociated mice is in order.

Role of gut microbes in regulating oral tolerance

Although the systemic and mucosal immune systems are somewhat discrete, it seems that Ag in the gut lumen can affect elements of both systems. A profound effect of gut Ag is to induce “oral tolerance.” Oral tolerance is a phenomenon that has most convincingly been defined by the oral ingestion/administration of protein (“dietary”) Ag, resulting in a decreased systemic response to local “priming” with the Ag, as reflected in diminished *in vivo* or *in vitro* response to specific Ag challenge. Usually, the diminished response assessed is of Th1 cells or of T-dependent systemic antibody responses (Dahlman-Hoglund *et al.*, 1995; Lundin *et al.*, 1996; Alpan *et al.*, 2001). Presently, oral tolerance itself is considered by most as a positive, specific immune response that has down-regulatory consequences for these previously mentioned aspects of the multifaceted immune response. One opera-

tionally defined lymphoid mediator of this downregulatory effect has been termed Tr1 (or Treg) cells, which can be described as follows: CD4⁺ T cells that are CD25⁺, produce IL-10 and/or TGF- β , disseminate from the gut mucosa, proliferate only minimally or not at all *in vitro* to Ag/APC, and are capable of downregulating responses *in vivo* to Ag corresponding to their own specificity as well as to irrelevant Ag that are stimulating other specific T cells in their presence (bystander effect) (Dahlman-Hoglund *et al.*, 1995; Thorstenson and Khoruts, 2001; Lundin *et al.*, 1999; Shevach, 2001; Chen *et al.*, 1998).

One of the first examples of the effects of the gut microflora on the development of oral tolerance was given by Wannemuehler *et al.* (1982), who used feeding of sheep erythrocytes, a classical oral toleragen (Mattingly and Waksman, 1978), to show that convincing oral tolerance developed in *CNV* but not *GF* mice. More recently, this finding has been extended to use of ovalbumin as an oral toleragen by Moreau *et al.* (1999), and this group also showed that formerly *GF* mice monoassociated with *E. coli* did develop oral tolerance to ovalbumin while similar mice monoassociated with bifidobacteria did not. This latter report highlighted the problems of achieving effective systemic priming in Trexler-type isolators; this step almost precludes the use of parenterally administered Ag in CFA. We have overcome this problem by using maleylated (MA) Ag (Singh *et al.*, 1998), which can be filter-sterilized and easily introduced in glass ampules into the isolator. **Figure 18.7** shows the use of MA-Ag to compare the development of oral tolerance in *GF* versus *CNV* mice fed dietary Ag and the facilitation of oral tolerance upon monoassociating formerly *GF* mice with Schaedler's *E. coli*.

Presently, a principal question is how gut microbes affect the development of oral tolerance to dietary proteins. Kiyono *et al.* (1982) gave suggestive evidence of the role of a microbial product, LPS, in the induction of oral tolerance by showing that LPS-responder mice, such as C3H/HeN-strain

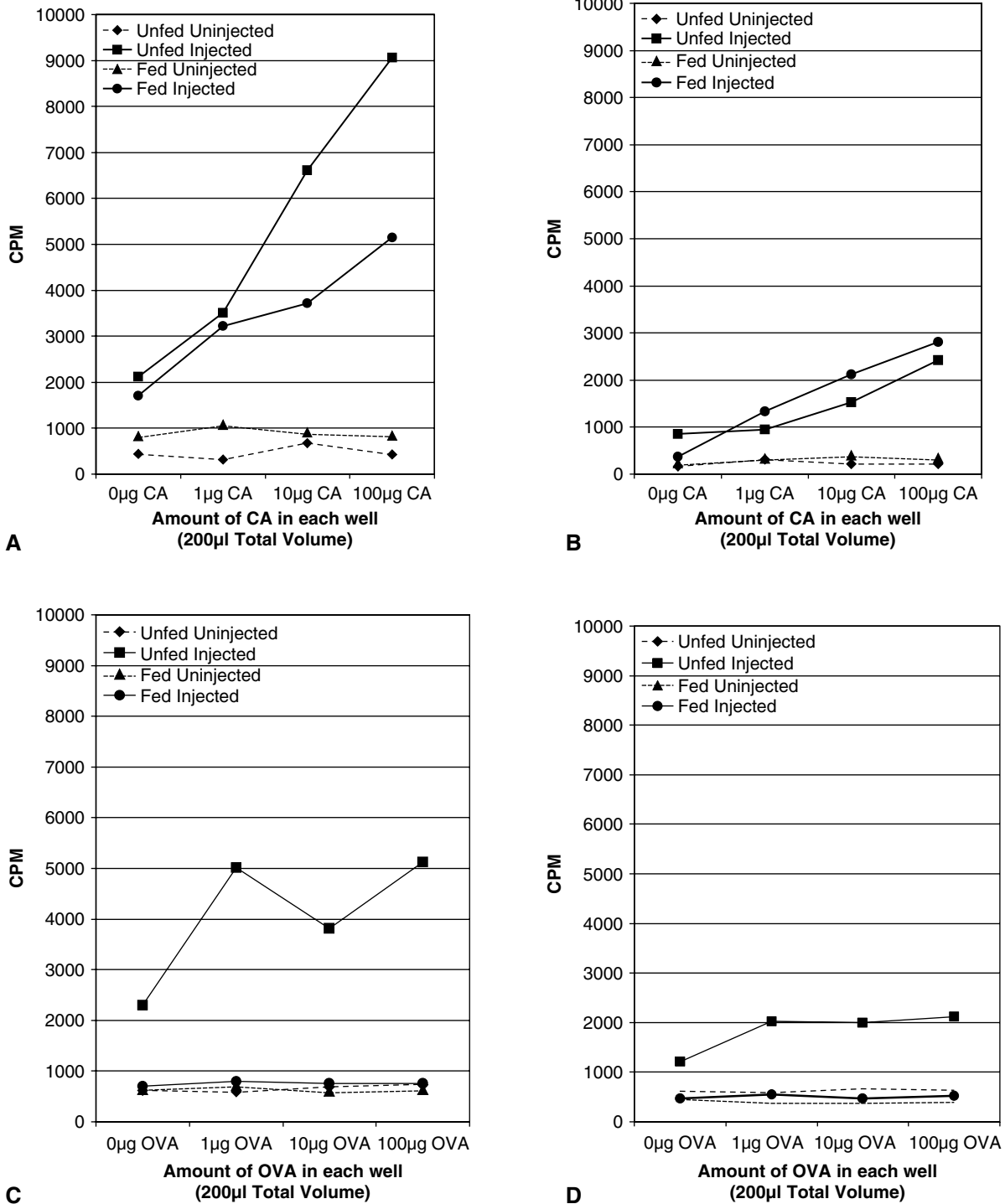


Fig. 18.7. Proliferation data with use of β -emission from 3H-thymidine incorporated into mouse spleen cells cultured for 4 days with varying concentrations of proteins. **A**, CNV mice fed, injected, and cultured with conalbumin (CA); **B**, GF mice fed, injected, and cultured with CA; **C**, CNV mice fed, injected, and cultured with OVA; **D**, *E. coli*-monoassociated mice fed, injected, and cultured with OVA (George, A., Boiko, N., and Cebra, J., unpublished data).

mice, developed prolonged and profound oral tolerance to sheep erythrocytes, while LPS-nonresponder mice (C3H/HeJ) did not. However, Moreau and Cortthier (1988) found that GF C3H/HeJ mice did develop convincing oral

tolerance after feeding with ovalbumin, but it was maintained for a shorter period than in CNV C3H/HeJ mice. Perhaps microbial products other than LPS, found in mice with a complete microflora, can facilitate oral tolerance. Why

GF C3H/HeJ mice do exhibit oral tolerance, albeit for a shorter duration than their CNV counterparts (Moreau and Corthier, 1988), while LPS-responder GF mice do not (Moreau *et al.*, 1999; Fig. 18.6) is unclear.

The development of “suppressor T cells,” now called *downregulatory* or *Tr1* cells, that disseminate systemically and mediate oral tolerance has been recognized for many years (Mattingly and Waksman, 1978). It seems likely that these *Tr1* cells depend upon Ag presented by interdigitating DC for their selective development, possibly including those that extend through the IEL spaces and interface with the gut lumen (Viney *et al.*, 1998; Akbari *et al.*, 2001; Alpan *et al.*, 2001; Maric *et al.*, 1996; Rescigno *et al.*, 2001). Since Caramalho *et al.* (2003) have recently shown that LPS can selectively activate *Tr1* cells via their TLR and that this activation is synergistic with activation via TCR, it remains to be shown whether particular protein Ag from enteric bacteria can possibly activate *Tr1* cells via their TCR and whether various microbial products, such as LPS, can synergize this activation via TLR. To involve such a mechanism to explain the effects of gut bacteria on the development of oral tolerance would require that *Tr1* cells act in a bystander fashion in the periphery, but this has already been demonstrated (Dahlman-Hoglund *et al.*, 1995; Lundin *et al.*, 1996).

Finally, here we address whether mice and rats become orally tolerant, via the gut mucosal, to members of their normal gut microbiota and, if so, when they develop this oral tolerance. Perhaps the most informative experiments performed thus far involve the comparison of rats colonized at birth or at 6 weeks of age with an *E. coli* expressing plasmid-encoded ovalbumin. Streptomycin/ampicillin was given prior to oral colonization of young rats and near-term pregnant dams, and administration of ampicillin was continued via the drinking water of both young rats and naturally colonized neonates. The latter showed oral tolerance to both ovalbumin and pillus Ag when challenged 6 weeks after colonization, while the adults exhibited an exaggerated systemic response, indicative of oral priming (Karlsson *et al.*, 1999). In a mouse model, adults displayed oral tolerance after oral administration of sonicates of *Leishmania major* and *Staphylococcus aureus*; however, similar treatment with sonicates of *E. coli* and *Salmonella typhimurium* indicated no development of oral tolerance but development of priming for an exaggerated response to challenge (Garg *et al.*, 1999). More work needs to be done to assess whether commensal microbes induce specific, *Tr1*-mediated oral tolerance and why the time window seems to be restricted to the postnatal period. It is also not clear whether GF young adults may or may not be orally tolerized versus members of the normal microbiota upon monoassociation or oligoassociation.

Mechanisms for the interactions of commensal bacteria with intestinal epithelial cells and dendritic cells and consequent effects on lymphoid elements

The complex microbiota in mammals is sequentially developed after birth and dynamically maintained throughout life (Savage, 1999). Evolutionarily, commensal relationships are

established through host-microbial interactions. At the front line of the interactions are the mucosal surfaces, which are in direct contact with varieties of members of microbiota. In the gastrointestinal tract, the cross-talk between mucosal microbiota and intestinal epithelial cells is physiologically significant in maintaining the homeostasis of the microecosystem. Yet little is known about the interplay and how it influences the physiology of the gastrointestinal tract.

Because of the complexity of the microbiota (about 400 bacterial species, the majority of which are obligate anaerobes) and the difficulty in maintaining live primary gut ECs *ex vivo*, most of our limited understanding of microbial-EC interactions is entirely based on the information obtained from *in vitro* culture of cell lines with pathogenic bacteria (Elewaut *et al.*, 1999; Eckmann *et al.*, 2000). Gnotobiotic animals provide a unique model for investigating host-microbial interactions *in vivo* (Gordon and Pesti, 1971). This valuable approach generally starts with a comparative study of conventionally reared and GF animals. To further pinpoint the member(s) of microbiota that may be responsible for certain effects on the host cells, the dissected member(s) of microbiota are further examined by analyzing formerly GF animals associated with a single bacterium or a defined group of bacteria and comparing these with GF and CNV reared animals. There are two general approaches to profiling gene expression upon host-microbial interactions (Kagnoff and Eckmann, 2001). One approach is to identify differentially expressed mRNAs and then further define the identity of the respective genes and their function. The other approach is to analyze global gene expression by assessing changes in large numbers of defined genes with DNA microarray technology. The latter is a powerful tool for discovery-oriented studies of host-microbial interactions, as a broad range of host cell activities are monitored. New discoveries through such analyses may serve as guidance for further elucidating the function and significance of the differentially expressed genes. Combined with the technology of laser-capture microdissection, which can isolate a single cell type from a complex tissue sample, it becomes possible to characterize the effects of members of microbiota on a particular host cell population (Stappenberg *et al.*, 2002a).

Role of intestinal microbiota in the development and physiology of the intestine

The pioneering work on this subject had showed the importance of intestinal microbiota in the development of normal morphology and function of the gastrointestinal tract (Gustafsson *et al.*, 1982). Compared to conventionally reared counterparts, GF rodents showed a greatly enlarged cecum, thinner intestinal wall, reduced overall cell mass, and less IgA plasma cells in the gut (Gordon and Pesti, 1971; Thompson and Trexler, 1971; Crabbe *et al.*, 1968). Without microbiota, the EC cycle is significantly changed, being characterized by slower epithelial renewal, lower mitotic index of crypt ECs, and prolonged transit process of EC differentiation (Abrams *et al.*, 1963; Leshner *et al.*, 1964; Cook and Bird, 1973). The impact of intestinal microflora on other aspects

of intestinal function has also been observed. GF rodents showed reduced intestinal motility (Abrams and Bishop, 1967) and distinct patterns of enzyme production by ECs, different from those of CNV mice (DeBoth and Plaisier, 1974).

In recent years, advances of modern technology have led to a fine example of intestinal EC-commensal bacterial interactions (Bry *et al.*, 1996). As a sensitive marker of gut epithelial differentiation, fucosylated glycoconjugate expression on ECs has been monitored and compared in GF versus CNV reared neonatal mice. Intestinal microbiota are required for the production of fucosylated glycoconjugate on intestinal ECs, as it was not expressed in GF mice. However, the expression could be initiated in formerly GF young adult mice upon monoassociation with *Bacteroides thetaiotaomicron*, a member of the microbiota in the ileum of humans and rodents. The production of fucosylated glycoconjugates was associated with accumulation of α 1, 2-fucosyl-transferase gene transcripts in ECs. An interesting finding is that the production of fucosylated glycoconjugates depended on the density of the bacterial population and the ability of the colonizer to use fucose as a carbon source, as isogenic strains of *B. thetaiotaomicron* that had disrupted fucose utilization regulon were not able to induce ECs to produce fucosylated glycoconjugates. Umesaki *et al.* (1995) have previously shown that another single indigenous intestinal bacterium, SFB, could induce production of fucosyl asialo GM1 glycolipids on small intestinal ECs in monoassociated formerly GF mice, similar to that observed in conventionalized mice. With use of DNA microarray, analysis of *B. thetaiotaomicron*-monoassociated mice revealed effects of this single member of the microbiota in modulating gene expression involved in multiple intestinal functions, including nutrient absorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis, and postnatal intestinal maturation (Hooper *et al.*, 2001). For instance, it was further demonstrated that *B. thetaiotaomicron*, like intestinal microbiota in CNV reared mice, could initiate the development of intestinal angiogenesis through interaction with Paneth cells in the intestinal epithelium and induce the development of normal intestinal vasculature in formerly GF mice (Stappenbeck *et al.*, 2002b). Moreover, a family member of mouse angiogenins, angiogenin 4, which is a member of the RNase superfamily like other angiogenins, was found to have microbicidal activity and be specifically produced and secreted into the gut lumen by Paneth cells in the crypt of the small intestine (Hooper *et al.*, 2003). The expression and secretion of angiogenin 4 were induced and regulated by commensal bacteria and bacterial products, such as *B. thetaiotaomicron* and LPS in the intestine, representing one of the mechanisms by which the balance or homeostasis of the microecosystem is maintained through the delicate host-microbial interactions. Most recently, another study showed that a colon-specific gene expression that encoded for the production of a secreted protein called RELM β was regulated by microbial colonization in the gut, as conventionalized formerly GF mice had dramatically enhanced expression and secretion of RELM β into the stool in comparison with the very low level

prior to the introduction of the normal microbiota (He *et al.*, 2003). In mice, RELM β was produced specifically by goblet cells throughout the large intestine, with the highest level in the cecum and the distal half of the colon. The mRNA for RELM β was solely expressed in the crypt epithelium, suggesting the activation of the RELM β gene occurs in the crypt. It is not known yet which specific member(s) of the microbiota or their products induce RELM β expression and what the significance is of the RELM β production in the large intestine.

The profiles of the host gene-expression changes modulated by various indigenous members of microbiota were somewhat different. It is likely that the outcome of the host-microbial interactions under conventional conditions results from the collective effects of the members of microbiota, of which the composition is continuously varying.

Impact of the interactions between intestinal microbiota and ECs on innate and adaptive immunity

It becomes apparent that the dialogue between intestinal microbiota and ECs has great impact on innate and adaptive immunity. Intestinal ECs are more actively participating in regulating immune responses beyond just being a passive barrier at the interface between external and internal environment (Kato and Owen, 1999). It has been demonstrated that intestinal ECs constitutively express several members of transmembrane receptors called toll-like receptors (TLRs) (Cario *et al.*, 2002). TLR family members recognize and respond to different microbial-associated molecular patterns (Barton and Medzhitov, 2002). Recognition by TLRs initiates a signaling pathway that leads to activation of NF- κ B transcription factors and members of the MAP kinase family, thereby initiating the innate immune response (Medzhitov, 2001). The transcription factor NF- κ B is a major player in the inflammatory immune response of the gut (Tak and Firestein, 2001). Activation of NF- κ B in intestinal ECs by proinflammatory stimuli or pathogenic bacteria results in expression of various genes encoding inflammatory cytokines and chemokines (Elewaut *et al.*, 1999; Gewritz *et al.*, 2000). A recent study showed that a nonpathogenic strain of *Salmonella* was able to interfere with the NF- κ B activation pathway elicited by diverse proinflammatory stimuli. Through direct interaction with model human epithelia, the nonpathogenic bacteria could block I κ B degradation, an inhibitor of NF- κ B, thereby abrogating synthesis of inflammatory mediators (Neish *et al.*, 2000). It is reasonable to speculate that this may also be the mechanism for the homeostatic state maintained at the intestinal mucosal surface under normal conditions (Xavier and Podolsky, 2000).

TLRs were also expressed on macrophages and DCs (Janeway and Medzhitov, 2002; Akira *et al.*, 2001), in which the impact of TLR signaling on homeostasis of the microecosystem may also be critical through innate immune responses and hence indirectly through adaptive immune responses. Moreover, recent studies showed that TLR signaling can serve as a direct link between the innate and adaptive immune system through TLRs expressed on B and T lym-

phocytes (Barton and Medzhitov, 2002; Matsuguchi *et al.*, 2000; Mokuno *et al.*, 2000). Furthermore, it was demonstrated that regulatory T cells (Tr1, CD4⁺CD25⁺) selectively expressed TLRs and could respond directly to proinflammatory bacterial products such as LPS, resulting in the increase of Tr1 survival/proliferation and the enhancement of their *in vitro* suppressive function (Caramalho *et al.*, 2003), which suggests that microbial products can directly modulate an adaptive immune response. Therefore, TLR signaling may represent another mechanism whereby the homeostasis of the microecosystem is maintained through host-microbial interactions.

Intestinal ECs can function as APCs and regulate T cell responses and other immune cells in the intestinal mucosa, thereby participating in adaptive immune responses (Kato and Owen, 1999). Besides the highly specialized M cells that overlie PP and can internalize and transport luminal Ags to the underlying lymphoid tissue, villous ECs are capable of uptaking and presenting antigen to T cells. Differing substantially from professional APCs, intestinal ECs can present unique and classical antigen-presenting molecules (classical and nonclassical MHC), costimulatory molecules (CD58, CD86), and some molecules that may facilitate antigen uptake (FcR γ , Ganglioside M1). In addition, most of these molecules are expressed in a polarized fashion, more on the basolateral or apical surface of the intestinal ECs, suggesting the complexity of ECs as APCs (Blumberg *et al.*, 1999; Hershberg and Mayer, 2000). These features enable ECs to interact with T cells in the mucosa. Overcoming the technical difficulties in isolating and maintaining primary intestinal ECs *ex vivo* for a relatively long period of time (14 days), Telega *et al.* (2000) showed that small intestinal and colonic ECs are both able to uptake protein Ag in a dose-dependent fashion, with a higher capacity shown by the latter. In comparison with wild-type mice, Ag uptake by colonic ECs from IL-2^{-/-} mice was increased, in parallel with higher expression of MHC class II molecules. Consistent with the increased levels of Ag uptake, Ag-pulsed colonic ECs from IL2^{-/-} mice were able to induce the highest levels of specific T cell activation in an MHC class II restricted way. Very little is known about how intestinal ECs, as APCs, deal with the tremendous amounts of Ags from intestinal microbiota and modulate immune response at mucosal sites. Indigenous bacteria, such as SFB, can induce expression of MHC class II molecules on small intestinal ECs and strongly stimulate the mucosal immune system (Umesaki *et al.*, 1995; Klaasen *et al.*, 1993; Talham *et al.*, 1999). SFB are in intimate contact with intestinal ECs, and the attachment induces apparent morphological changes at the apical surface of ECs, including indented, thickened, and more electron-dense membrane and accumulation of actin filaments (Davis and Savage, 1974; Jepson *et al.*, 1993). Recently, Yamauchi and Snel (2000) have presented evidence strongly suggesting that SFB are phagocytosed and intracellularly processed in intestinal ECs of chicken ileum. Alternatively, according to the findings of Rescigno *et al.* (2001), SFB may also be sampled into the LP from the surface of the epithelium by the protruding

dendrites of the DCs from the LP, which can open the tight junction between the ECs and meanwhile are able to express tight-junction proteins to preserve the integrity of the epithelial barrier. In addition, it was recently observed that porcine SFB could pass by M cells to the subepithelial region, which overlies PP (Meyerholz *et al.*, 2002). These findings may show part of the mechanisms by which SFB act as a potent stimulus of the mucosal immune system. Through interactions with normal microbiota at the front line, ECs may be an important modulator for maintaining homeostasis and for preventing a pathological inflammation.

Probiotics

Confronted by increasing amounts of antibiotics over the past 60 years, bacteria have responded with the propagation of progeny no longer susceptible to them. We currently face multiresistant infectious disease organisms that are difficult and sometimes impossible to treat against successfully. Today we can list a number of organisms in both hospitals and the community that thwart treatment because they are resistant to not one but many different antibiotics. The term “multidrug resistance,” which initially described resistant mammalian tumor cells and later strains of *Mycobacterium tuberculosis*, now can describe *any* multiresistant microorganism—bacterium, fungus, or parasite (van der Waaij and Nord, 2000). Among these opportunistic pathogens are the enterococci, the coagulase-negative staphylococci, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Clostridium perfringens*, and *Acinetobacter baumannii*. Those physicians attending medical school 20–30 years ago probably did not even discuss these organisms as important pathogens, although today they cause prominent, even potentially lethal problems in hospitals worldwide.

The majority of research to date has focused on the mechanisms by which pathogenic bacteria achieve their detrimental effects. It was even possible to develop a new infectious model by using a hypertoxigenic enterohemorrhagic *E. coli* strain in GF mice (Taguchi *et al.*, 2002). However, more recently, attention has turned to the indigenous nonpathogenic microorganisms and the ways in which they benefit the host (Hart *et al.*, 2002). The mammalian intestinal tract contains a complex, dynamic, diverse society of nonpathologic bacteria. Indeed, the number of bacteria that colonize the human body is so large that researchers have estimated that the human body contains 10¹⁴ cells, only 10% of which are not bacteria and belong to the human body proper (Teitelbaum and Walker, 2002). Further understanding of the beneficial effects of developing a normal bacterial flora is achieved by the analysis of GF animal models.

Documentation of the health benefits of bacteria in food dates back to as early as the Persian version of the Old Testament (Genesis 18:8), which states “Abraham owed his longevity to the consumption of sour milk.” Plinius, a Roman historian in 76 B.C., recommended the use of fermented milk products for the treatment of gastroenteritis (Schrezenenmeir and deVrese, 2001). In 1908 Nobel Prize-winning Russian scientist Elie Metchnikoff suggested

that the ingestion of lactobacillus-containing yogurt decreases the number of toxin-producing bacteria in the intestine and thus contributes to the longevity of Bulgarian peasants (Metchnikoff, 1907; Sanders, 2000). He proposed that the ingestion of saccharolytic organisms in the form of fermented milk was reversing the effect of the proteolytic organisms, which caused auto-intoxication of the host. He isolated microorganisms from fermented milk. One strain he called the Bulgarian bacillus (this was *Lactobacillus bulgaricus*, which is now called *L. delbrueckii* subsp. *bulgaricus* and with *Streptococcus salivarius* subsp. *thermophilus* is used to ferment milk to yogurt).

It was these observations that led to the concept of a “probiotic,” derived from the Greek, meaning “for life.” The term was first used (Lilley and Stillwell, 1965) in contrast to the word antibiotic and defined as “a substance secreted by one microorganism, which stimulates the growth of another.” Later (Sperti, 1971) this word was used to describe tissue extracts that stimulate bacterial growth. The origin of the term (as microbial feed/food supplement) is attributed to Alan Parker (Parker, 1974), who defined probiotics as “organisms and substances which contribute to intestinal microbial balance.” Roy Fuller (1989) modified this term as a “live microbial feed supplement which beneficially affects the host animal or human by improving its intestinal microbial balance.”

A probiotic should

- be of animal host origin (the attachment to ECs is very host-specific, which means in practical terms that a strain that is suitable for development in one animal may not be active in another);
- be nonpathogenic in nature;
- be resistant to destruction by technical processing;
- be resistant to destruction by gastric acid and bile;
- adhere to intestinal epithelial tissue;
- colonize the gastrointestinal tract, if even for a short time;
- produce antimicrobial substances;
- modulate immune responses; and
- influence gut metabolic activities (e.g., cholesterol assimilation, vitamin production) (Gibson and Fuller, 2000).

According to Roy Fuller (1989), the species currently being used as probiotics include the following: *L. bulgaricus*, *L. acidophilus*, *L. plantarum*, *L. casei*, *L. helveticus*, *L. lactis*, *L. salivarius*, *S. thermophilus*, *Enterococcus faecium*, *E. faecalis*, *Bifidobacterium* species, and *E. coli*.

Some commercial probiotic preparations contain a *Bacillus subtilis* strain or *B. cereus* as one of their components (Kirchgessner *et al.*, 1993; Hoa *et al.*, 2000). Therefore, *B. subtilis* and *B. cereus* are two *Bacillus* species that may have potential value as probiotics (Maruta *et al.*, 1996). Other microorganisms also can be used or considered for use as probiotics in humans or animals, including various *Bacteroides* species, *Propionibacterium* species (Zarate *et al.*, 2002), and fungi. For example, *Saccharomyces boulardii* is a patented yeast preparation that has been shown to inhibit the

growth of pathogenic bacteria both *in vivo* and *in vitro*. It can prevent antibiotic-associated diarrhea, live at an optimum temperature of 37°C, be resistant to digestion, and thus reach the colon in a viable state. However, once therapy is completed, the yeast is rapidly eliminated (Surawics *et al.*, 1989).

Probiotics in veterinary practice

We shall consider only certain of the most promising microorganisms as efficacious probiotics. Oral dosage of poultry with native gut microorganisms to prevent infection with *Salmonella* was first examined by Nurmi and Rantala (1973). Prophylactic inoculation of GF chicks with *L. acidophilus* was shown to reduce shedding of pathogenic *E. coli* from 100% to 47% (Watkins *et al.*, 1982). For instance, the use of an avian-specific probiotic containing *L. acidophilus* and *S. faecium* for reducing the shedding and colonization of *Clostridium jejuni* in the chicken intestinal tract has been evaluated (Morishita *et al.*, 1997). Reducing (to 27%) the *C. jejuni* colonization level in chickens can potentially decrease the incidence of *C. jejuni* infections in humans. Nowadays, more attention is being paid to selection of promising probiotic strains according to the criteria mentioned previously. From a total of 112 strains of lactic acid bacteria of duck origin, only two—*L. animalis* and *L. salivarius*, on the basis of tests for aggregation, coaggregation, tolerance to acidic pH, detection of inhibitory activity, characterization of adhesion, and *in vivo* persistence—were proposed for use in poultry (Ehrmann *et al.*, 2002). It is interesting that 18 of 1200 bacterial isolates from cattle feces and intestinal tissue samples, which were screened and determined to inhibit the growth *E. coli* O157:H7 *in vitro*, were identified as other strains of *E. coli*, and one other strain was *Proteus mirabilis*. All 19 of these strains are nonproducers of Shiga toxin. Those selected probiotic bacteria, orally administered to cattle prior to exposure to *E. coli* O157:H7, can reduce the level of carriage of *E. coli* O157:H7 in most animals (Zhao *et al.*, 1998). Another set of data suggests that *L. salivarius* 51R, which was isolated from chicken caeca and administered orally to newly hatched broiler chickens, can significantly lower counts of enterococci and coliforms in the crop during the whole experimental period (Rada and Rychly, 1995). Recently presented results (La Ragione *et al.*, 2001) confirm that a single oral inoculum of 2.5×10^8 *B. subtilis* spores sufficiently suppressed all aspects of infection with *E. coli* O78:K80 (a known virulent strain associated with an avian colibacillosis).

Gnotobiotic and GF studies: models and approaches

In CNV animals, a study of the specific role of microorganisms as well as their interactions is difficult to achieve, given the complexity of the microbial ecosystem of the gastrointestinal tract. Although numerous data have been published on the dynamics of different representative constituents in CNV young, still little is known concerning gnotobiotic animals with a limited and defined microflora of the digestive tract. However, it has long been appreciated that the levels of

several biochemical indices in the blood as well as immune responses of CNV versus gnotobiotic animals differed substantially. Only four various species of bacteria (but five strains) isolated from rumen of adult sheep—*S. bovis* C277, *Prevotella ruminicola* B14, *Butyrivibrio fibrisolvens* SH1, *B. fibrisolvens* JW2, and *Selenomonas ruminantium* Z108, which each were used in quantities of 1×10^9 microbial cells for inoculation of GF lambs—can significantly influence the level of the digestive processes as well as intensiveness of nutrient metabolism (Bomba *et al.*, 1995). In general, these results enable us to state that differences in the ecosystem of the digestive tract influenced both nitrogen and energy metabolism in CNV and gnotobiotic lambs. Rumen contents of gnotobiotic lambs showed significantly higher α -amylase and cellulase activities than in CNV lambs. This finding could be due to the composition of the inoculum, which presented a uniform population of amylolytic and cellulolytic bacterial strains. Since the ecosystem of the CNV animals contained a whole range of metabolic groups of microorganisms, the proportions of amylytic and cellolytic bacteria are decreased through competition. Total serum protein levels and urea levels in CNV animals were also increased. Glucose levels did not differ significantly between groups. Total lipids in gnotobiotic lambs were substantially elevated. These differences between the groups persisted until the end of the investigation, although daily weight gains were similar. Another set of experimental data suggests that inoculation only by a single bacterium (*L. casei*) in gnotobiotic lambs (Bomba *et al.*, 1997), as in gnotobiotic chicks (*L. acidophilus*) (Watkins *et al.*, 1982), can protect against enterotoxigenic *E. coli* infection. Three other species (*L. acidophilus*, *Saccharomyces boulardii*, and *E. coli*) each are individually used (Figueiredo *et al.*, 1996) also as probiotic strains to protect against enteropathogenic agents. In order to determine if there was a synergistic effect of individual microbes, organisms were orally administered to GF mice (*L. acidophilus* and *E. coli* intragastrically in a single dose of 10^8 viable cells; *S. boulardii* orally, every 2 days throughout experiment, 10^9 viable cells at each administration). Ten days after colonization of the digestive tract, groups of animals associated with these microorganisms or not (control) were challenged orally with streptomycin-resistant or -susceptible strains of *Shigella flexneri* or *S. enteritidis* subsp. *typhimurium*. All possible diassociations and monoassociations of the three probiotics with gnotobiotic mice were also performed, and these experiments led to the conclusion that only in mice in which *E. coli* was present could antagonism be obtained (Figlo-Lima, 2000).

Probiotics in medicine

The presence of the indigenous flora is crucial not only for maturation of the immune system but also for the development of normal intestinal morphology and maintenance of a chronic and immunologically balanced intestinal inflammatory response—so-called physiological inflammation. Probiotics appear to be useful in the prevention or treatment of several gastrointestinal disorders of humans, including infectious diarrhea, antibiotic diarrhea, and traveler's diarrhea

(Salminen *et al.*, 1996; Gionchetti *et al.*, 2000). Results of preliminary human studies suggest that patients with IBD and even irritable bowel syndrome may benefit from probiotic therapy (Vanderhoof and Young, 1998). Most important is that each proposed probiotic must be studied individually and extensively to determine its efficacy and safety in each disorder for which its use may be considered. A double-blind, placebo-controlled study has shown that treatment with *Lactobacillus GG*, administered orally, was able to significantly reduce the incidence and duration of rotavirus diarrhea in comparison with a placebo (Isolauri *et al.*, 1991). Saavedra *et al.* (1994) have shown that supplementing an infant formula with *Bifidobacterium bifidum* and *S. thermophilus* can reduce the incidence of acute diarrhea and rotavirus shedding in infants admitted to the hospital. Only two other examples are known of probiont bacteria—*Lactobacillus GG* (Biller *et al.*, 1995) and *S. boulardii* (McFarland *et al.*, 1994)—successful in the treatment of diarrhea in relapsing colitis due to *Clostridium difficile*. Oral bacteriotherapy with *Lactobacillus GG* in Crohn's disease indicates that such probiotic bacteria may have the potential to increase gut IgA and thereby promote the gut immunological barrier, irrespective of the course of the main disease (Malin *et al.*, 1996). Probiotics have been examined and therefore reviewed (Rolfe, 2000) for their effectiveness in the prevention and treatment of other gastrointestinal disorders such as antibiotic-associated and infectious diarrhea (including those caused by *Shigella*, *Salmonella*, enterotoxigenic *E. coli*, and *Klebsiella*) and *H. pylori* gastroenteritis. It is interesting that *Lactobacillus* has been shown to be antagonistic to *H. pylori* both *in vitro* and in a gnotobiotic murine model (Kabir *et al.*, 1997; Aiba *et al.*, 1998). Unfortunately, the results of a few studies involving *Lactobacillus* and humans are conflicting.

Two really promising practical applications of *E. coli* non-pathogenic strain O83:K24:H31 (Lodinova-Zadnikova *et al.*, 1991, 2000, 2003) or O6:K5:H1 (Kruiz *et al.*, 1997) have been recently reported. *E. coli* serotype O6:K5:H1 was examined for its ability to prevent relapses of ulcerative colitis. Preliminary data look promising and suggest that this may be another option in maintenance therapy for such diseases. The other *E. coli* strain was used in order to determine whether the common mucosal immune system was triggered (on tested inoculated healthy full-term infants) and to investigate effectiveness of artificial colonization of the intestine on the occurrence of nosocomial infections, presence of bacterial pathogens, and infant mortality (among high-risk infants in an intensive care unit). The most important advantage of this long-term presence of probiont bacteria in the intestine is probably stable protection of formula-fed infants from nosocomial infectious agents by an early induction of IgA antibodies that appeared in stool and saliva (partially compensating for their lack in maternal milk). In high-risk premature infants, preventive colonization was even more significant and resulted in a decrease in the number of infections, infant mortality, presence of pathogens in the intestine and other body locations, and need for antibiotics.

Clinicians should pay special attention to investigations of probiotic efficacy in the prevention of colon cancer. Studies

that have explored the cause–effect relationship directly have used animal models. The general conclusion of these studies is that probiotics have an inhibitory effect on the development of aberrant crypts and tumors (Brady *et al.*, 2000).

Two final examples of successful use of probiotics are the treatment of food-allergy diseases (Paganelli *et al.*, 2002) and the prevention of atopic diseases such as eczema, allergic rhinitis, and asthma (Kalliomaki *et al.*, 2001).

Postulated mechanisms of probiotic activity

Possible mechanisms of probiotic activity are still mostly enigmatic. However, nowadays a largely accepted hypothesis is that effective probiotics initiate a complex, multifaceted process. For example, proven phenomena of so-called colonization resistance (the sum of all processes by which benign bacteria can inhibit the colonization by other mainly pathogenic strains) could not explain the mechanisms of action of particular probiotics against particular pathogens. Sometimes the same probiotic may inhibit different pathogens by different mechanisms. Here are brief descriptions of some nonspecific and specific mechanisms by which probiotics can protect the host against various diseases.

One mechanism involves the production of inhibitory substances against both gram-positive and gram-negative microorganisms (organic acids, especially produced by *Lactobacillus* species as metabolic substances; hydrogen peroxide and bacteriocins). Antibiotics, as substantially important products of probiotic-based bacteria, can also be responsible for their antibacterial properties (Pinchuk *et al.*, 2001). *B. subtilis* strain 3 is known to have antagonistic properties against some species of the family Enterobacteriaceae, but it has been shown to also inhibit *H. pylori* *in vitro*. One of the compounds of cell-free supernatant from *B. subtilis* to which *H. pylori* cultures were susceptible was identified as amicumacin A, an antibiotic with anti-inflammatory properties.

A second mechanism involves the blocking of adhesion sites. Competition for bacterial adhesion sites on intestinal epithelial surfaces is a proven mechanism of action for probiotics, which is why it is so important to select potentially effective strains according to their ability to adhere to host epithelial cells.

Competition for nutrients has also been proposed as a mechanism of probiotic action, but *in vivo* evidence of this is lacking (Rolfe, 2000).

A fourth mechanism is degradation of toxin receptor. The best example of this is *S. boulardii*, which can protect animals against *C. difficile* intestinal diseases through degradation of the toxin receptor on the intestinal mucosa (Castagliuolo *et al.*, 1996, 1999). Properties of some probiont bacteria to neutralize the effect of enterotoxins were also mentioned as possible mechanisms of their activity (Sissons, 1989; Boiko, 2000).

Fifth is stimulation of immunity. Recent evidence suggests that stimulation of specific as well as nonspecific systemic and secretory immunity may be a basic mechanism by which probiotics can protect the host, especially against

enteropathogens (Perdigon *et al.*, 1995; Cukrowska *et al.*, 2002). The underlying mechanisms of immune stimulation are not well understood yet, but specific cell wall components or even cell layers may act as adjuvants and significantly increase humoral immune responses. An interesting observation was that *L. casei* induced translocation of the normal flora to the liver in malnourished mice. Usually treatment with *Lactobacillus* or *Bifidobacterium* (Yasui *et al.*, 1992) was associated with an enhancement of specific antibody-secreting cells to pathogens. Therefore, it was suggested that certain strains of lactic acid bacteria, particularly *Lactobacillus GG*, promote both systemic and local immune responses. It is interesting that comparison of the immunological effects of viable and heat-inactivated lactic acid bacteria (Majamaa *et al.*, 1995) shows that *Lactobacillus GG*, administered as a viable preparation during acute rotavirus gastroenteritis, resulted in a significantly increased rotavirus specific IgA response at convalescence; the heat-inactivated form was clinically as efficient, but the increased IgA response was not detected. Probiotic modulation of humoral, cellular, and nonspecific immunity has been generally reviewed (Erickson and Hubbard, 2000). In addition, recently it was shown that *B. bifidum* but not *C. perfringens* can significantly induce total IgA and IgM synthesis by both MLN and PP mucosal B cells. Mucosal antibody production following oral administration of *B. bifidum* to mice caused increased numbers of IgG, IgM, and IgA in the culture of splenic cells, but such bacteria did not induce their own specific antibody response.

Recently it has been shown that a human intestinal microflora strain, *Lactobacillus GG* (ATCC 53103), promotes local antigen-specific immune responses (particularly of the IgA class), prevents permeability defects, and confers controlled antigen absorption. In that study the concentration of fecal α 1-antitrypsin, tumor necrosis factor α , and eosinophil cationic protein were determined as markers of intestinal inflammation before and after probiotic intervention. The clinical score of atopic dermatitis improved significantly in infants treated with *Lactobacillus GG*. The concentration of α 1-antitrypsin decreased in this group, but not in the group without treatment. In parallel, the median (lower-quartile to upper-quartile) concentration of fecal TNF- α decreased significantly in this group in comparison with those receiving the extensively hydrolyzed whey formula only. The concentration of fecal eosinophil cationic protein remained unaltered during therapy. These results suggest that probiotic bacteria may promote endogenous barrier mechanisms in patients with atopic dermatitis and food allergy, and by alleviating intestinal inflammation, they may be a useful tool in the treatment of food allergy (Majamaa and Isolauri, 1997).

Finally, some protective mechanisms of probiotics in the development of colon tumors have been postulated: prevention of mutations and antigenotoxic activity (decreased DNA damage in colon cells); decreased procarcinogenic enzyme activity; binding of mutagens and/or a decrease in their excretion; and decrease in the proliferation of transformed cells and their apoptosis (Wollowski *et al.*, 2001).

Involvement of infectious components present on mucosal surfaces in etiology and pathogenic mechanisms of idiopathic, inflammatory, and autoimmune diseases

While the major cause of death in the less-developed world remains infectious diseases, the major killers in the developed world are cardiovascular disease and cancer. High morbidity is caused by chronic disorders such as allergy, arthritic diseases, and other inflammatory and autoimmune diseases.

The main characteristics of inflammatory and autoimmune diseases are tissue destruction and functional impairment as a consequence of immunologically mediated mechanisms that are principally the same as those functioning against dangerous (pathogenic) infections. In the case of autoimmune diseases, a major effort has been made to understand the pathogenic mechanisms leading to the loss of tolerance to self-components (autoantigens) (Bach, 2002; Shoenfeld *et al.*, 2002).

Despite the fact that target antigens and the genetic bases of several autoimmune diseases are now better understood, the initial events leading to a loss of tolerance toward self-components remain unknown. One of the most popular explanations for autoimmune phenomena has always centered on various infections as possible natural events capable of initiating the process in genetically predisposed individuals (Tlaskalova-Hogenova *et al.*, 1998; Rose and Mackay, 2000; Shoenfeld *et al.*, 2002). Increased interest in infectious agents as causes of chronic diseases was awakened by the discovery of *H. pylori* as a causative agent of stomach ulcers, chronic gastritis, and probably gastric cancer.

A number of defined microorganisms have been shown to evoke autoimmunity. Infection with intestinal microbial pathogens such as *Salmonella*, *Shigella*, and *Yersinia* can trigger autoimmune reactions in joints and other organs (Toivanen and Toivanen, 2000). Diseases with autoimmune features such as rheumatic fever and acute glomerulonephritis may develop after a streptococcal infection. Also, viral infections can bring about autoimmune reactions; for instance, infection with coxsackievirus is accompanied by severe autoimmune myocarditis. Although there are several autoimmune disease models with well-defined initiating infections, for most autoimmune diseases the pathogenic role of plausible environmental agents is still being investigated.

The most accepted hypothesis about how infectious components cause autoimmunity is based on the concept of cross-reactive “molecular mimicry.” This hypothesis assumes a similarity between the epitopes of an autoantigen present in the afflicted organism and the epitopes in the environmental antigen. The latter may consist of a microorganism or another external antigen that causes the autoimmune response (Oldstone, 1987). Moreover, in identical twins, autoimmune disease does not necessarily develop in both individuals. This may be explained by a genetically based susceptibility to disease development and by changes in the immune regulatory mechanisms (“dysregulation”) as a consequence of environmental stimuli. With use of specific T-cell

clones and a broad spectrum of peptides derived from basic myelin protein, it was demonstrated that the activation of autoaggressive cells can be the consequence of a binding of structurally related but not necessarily identical peptides (Wucherpfennig, 2002). From this finding one can conclude that the stimulation of specific autoreactive cells can take place following a binding of structurally similar peptides originating from different environmental sources, *i.e.*, viral, bacterial, and/or food. Sequentially appearing responses to autoantigen epitopes or to autoantigenic molecules (epitope spreading) is a characteristic feature of developing autoimmune diseases. “Bystander” activation of immune cells was recently shown to be another mechanism by which autoimmune reactivity can spread.

Infectious stimuli may participate in the development of autoimmune conditions by inadequate activation of components of the innate immune system. Antigen-presenting cells, mainly DCs, could be activated (leading to maturation) by microbial components through TLRs to mediate the interaction of innate and adaptive immune systems. Adjuvant activity of microbial components or their synthetic substitutes has been found to correspond to the degree of activation of DCs. During activation of DCs, expression of costimulatory molecules increases, which can lead to changes in the presentation of self-antigens.

Increased synthesis and expression of heat stress proteins (hsp), chaperones, and transplantation antigens (molecules which could become target structures for autoimmune response) lead to abnormal processing and presentation of self-antigens by changing the transport and processing of intracellular peptides. Abnormal presentation of antigens can then evoke a response to cryptic self-epitopes, equal to the response to a dominant autoantigen. Superantigens are considered to be one of the most effective bacterial components to induce inflammatory reactions; they are believed to take part in the induction and course of autoimmune mechanisms (Oldstone, 1987; vanEden, 1991; Kotzin *et al.*, 1993; Tlaskalova-Hogenova *et al.*, 1998; Wucherpfennig, 2002).

The main contemporary approaches used to support the idea of the participation of microorganisms in the etiology of idiopathic, chronic, complex (polygenic) diseases are the following:

- Determination of prevalence of the disease in correlation with the occurrence of infection (epidemiology),
- Determination of antibodies directed to a certain (suspect) infectious agent in sera of patients (serology), and
- Detection of an infectious agent in blood or biopsy samples of the affected tissues by PCR or another sensitive method.

Unfortunately, the tests for identification of the infectious component are usually applied after diagnosis has been determined, that is, after a delay. This fact represents the main difficulty in characterizing the environmental triggering agent, because a long time has elapsed from the triggering event to the clinical onset of these idiopathic, chronic dis-

eases. Patients usually come to the clinic after their disease has become symptomatic and advanced, and this has made understanding of the early events leading to the disease very difficult. This is why experimental animal models of human diseases are used to elucidate the etiological and pathogenic aspects of these polygenic diseases.

In this connection it is interesting to note that experimental models of autoimmune disease induced by immunization with autoantigens practically all use complete Freund adjuvant containing mycobacteria (with strong immunostimulating activity) for immunization; moreover, some of them also use suspensions of killed bacteria, *e.g.*, *Bordetella pertussis*. Thus, for induction of autoimmune reactions in animals, it is necessary to activate DCs with microbial components to achieve activation of autoaggressive cells.

Genetically based or environmentally induced changes in mechanisms regulating mucosal immunity and tolerance can lead to impaired mucosal barrier function, increased penetration of microbial components into the circulation, and consequently, exaggeration of aberrant immune responses and inflammation. In fact, increased permeability of the gut mucosa was demonstrated in patients or their relatives with some autoimmune diseases (Yacyshyn *et al.*, 1996).

Some examples of chronic inflammatory and autoimmune diseases and the possible participation of infectious agents, including components from the normal microflora, in their etiology are presented next.

Inflammatory bowel diseases: IBD

Inflammatory bowel diseases (IBD) (*e.g.*, Crohn's disease and ulcerative colitis) are severe chronic disorders that affect approximately 0.2% of the human population. Despite the long-lasting interest of investigators, their etiology and pathogenesis remain unclear. IBD seem to involve interactions among immune, environmental, and genetic factors; the combination of these factors results in induction of inflammation, subsequent mucosal lesions, and then repair. Various specific microorganisms have been implicated in the etiology of these diseases.

Recently, experimental animal models of intestinal inflammation induced chemically or developing spontaneously have been described that make it possible to examine the early events (during induction) of the disease, to control all steps in disease progression, and to develop new preventive and therapeutic strategies. Several murine and rat models of spontaneously developing colitis suggest that disruption of T-lymphocyte regulatory functions or mucosal barrier defects could lead to chronic intestinal inflammation. Mice with a null mutation in the IL-2, IL-10, transforming growth factor β 1 (TGF- β 1), MHC class II, T-cell-receptor (TCR) α chain, and TCR- β chain and mice lacking signaling G protein subunit α -2 chain were shown to develop spontaneous chronic intestinal inflammation. When these mouse models of human disease were reared in GF conditions, the disease did not appear (Sadlack *et al.*, 1993; Hudcovic *et al.*, 2001; Kuhn *et al.*, 1993; Elson and Cong, 2002; Jiang *et al.*, 2002; Strober *et al.*, 2002). Therefore, it was suggested that dysreg-

ulation of the intestinal immune response to normal bacterial flora plays a crucial role. A loss of physiologically normal regulatory mechanisms of the local immune system, perhaps a breakdown of oral tolerance to environmental antigens (commensal gut bacteria), is probably involved in the pathogenic mechanism. Findings from experimental models of IBD indicate that T cells are responsible for the regulation of the intestinal immunological response to luminal antigens. Direct evidence of the participation of a subpopulation of CD4⁺ T cells in gut immunoregulation came from the finding that colitis in *scid* mice, developing after the transfer of a "pathogenic" CD45RB^{high} CD4⁺ T-cell subpopulation isolated from spleen of conventional BALB/c mice, could be prevented by simultaneous transfer of a "regulatory" CD45RB^{low} CD4⁺ T-cell population; these T cells express CD25 marker (Powrie, 1995). Surprisingly, the regulatory T-cell population isolated from GF mice was also able to suppress the inflammatory reaction when transferred together with pathogenic T cells (Singh *et al.*, 2001). In this model as well as in others, the important role of normal flora in disease development was confirmed, and the potential members of microflora responsible for intestinal inflammation were analyzed.

The finding that there is an abnormal T-cell responsiveness against indigenous microflora in human inflammatory bowel disease and its experimental models awakened interest in the possibility that commensal bacteria may initiate and/or maintain IBD lesions (Duchman *et al.*, 1995). Under conditions of an immunoregulatory disorder, the common intestinal flora is obviously capable of evoking stimulation leading to a chronic intestinal or systemic inflammation. A loss of physiological regulatory mechanisms of the local immune system or a lack of induction or breakdown of oral tolerance to commensal gut bacteria could be involved. The answer to the questions of which and how gut bacteria and which cells are involved in induction and maintenance of chronic intestinal inflammation is of great importance because it could bring about a new approach to therapy for and/or prevention of this severe disease (Xavier and Podolsky, 2000).

At present only three commensal enteric microbes have been shown to contribute to the initiation of IBD in each of three different animal models: *Bacteroides vulgatus* in HLA-B27 transgenic rats (Rath *et al.*, 1999); *H. muridarum* upon monoassociation of formerly GF *scid* mice then given CD45RB^{high}, CD4⁺ T cells (Jiang *et al.*, 2002); and *E. faecalis* in IL-10 (-/-) mice (Balish and Warner, 2002). Unfortunately, none of the effective strains of these bacteria has been used reciprocally to attempt to initiate IBD in the opposite animal model. The specificity or lack of specificity of any of these effective bacteria has not been defined; nor have the mechanisms for initiating CD4⁺ T cell-dependent IBD been clarified in any of these cases. Finally, there is some controversy as to whether *H. hepaticus* induces or potentiates colitis in IL-10 (-/-) mice (Dieleman *et al.*, 2000; Kuhlberg *et al.*, 1998). At any rate, *H. hepaticus* must be considered a frank pathogen, since it appears to initiate enterocolitis upon colonization of GF, *imcomp* mice (Fox *et al.*, 1996).

Periodontal disease

Periodontitis is a chronic inflammatory disease affecting the connective tissues and bone housing the teeth. The prevalence of the disease is high; a moderate form of this disease affects about 50% of adults, while the progressive, destructive form of periodontal disease occurs in a smaller proportion of the population (5%–15%). Periodontitis is a multifactorial disease in which immunological mechanisms, poorly understood genetic factors, and microbial etiology play a major role. The presence of subgingival flora is essential but not sufficient for the disease to occur. Periodontopathic bacteria that have been implicated in the etiology of the disease are *Porphyromonas gingivalis*, *Bacterioides forsythus*, *Actinobacillus actinomycetem comitans*, *Fusobacterium nucleatum*, and others (Seymour *et al.*, 1993; Henderson *et al.*, 1999; Wilson *et al.*, 2002). The possible involvement of these bacteria in periodontitis corresponds to the findings of increased levels of antibodies to these microbes in the patients and the pathological effects of these bacteria in animal models of this disease. Experimental models of the disease include GF rats monoassociated with *P. gingivalis*, mice infected with *P. gingivalis*, and ligated rat or hamster teeth. Most of these microorganisms are gram-negative bacteria, thus containing LPS that could induce polyclonal B cell activation. The essential role of bacterial involvement was demonstrated by the finding that induction of the disease by ligating the teeth was possible only in animals with an oral microflora. Periodontitis with bone loss did not occur in animals reared in GF conditions (Seymour *et al.*, 1993; Henderson *et al.*, 1999; Wilson *et al.*, 2002).

Rheumatic diseases

In recent years the relationship between the rheumatic diseases and the potential infectious components and elements of both innate and adaptive immune systems has been elucidated at the molecular level. Most forms of human diseases affecting connective tissues of the joints as well as experimental animal models of these diseases involve participation of microbial components. It is not only in reactive arthritis that intestinal bacteria were found to play the triggering role in disease development. The presence of microbial degradation products in the joint tissue was demonstrated in patients with rheumatoid arthritis, and the normal intestinal flora of these patients differs in composition from that of control healthy people (van Eden, 1991; de Keyser *et al.*, 1998; Rose and Mackay, 2000; Toivanen and Toivanen, 2000). In an animal model of human spondyloarthropathy, *i.e.*, in HLA-B27 transgenic rats or in BR10 mice, it was demonstrated that disease develops only in the presence of normal microflora; when these animals were kept in GF conditions, the disease did not develop (Rehakova *et al.*, 2000).

Type I diabetes, insulin-dependent diabetes mellitus (IDDM)

Insulin-dependent diabetes mellitus (IDDM) is one of the most studied organ-specific autoimmune diseases. It develops as a consequence of the selective destruction of β cells,

the pancreatic insulin-producing cells in the islets of Langerhans. It is generally accepted that autoimmunity against β cells may arise from activation of the immune system in genetically predisposed individuals by environmental factors bearing epitopes similar to those in the β cell. Candidate environmental antigens in IDDM include various microbial and food components. In particular, the most diabetogenic factors seem to be dietary proteins—in particular, cow's milk proteins. All these antigens are usually introduced to the body via mucosal surfaces. An increased risk for the development of IDDM has been associated with triggering of the immune system by enterovirus infection and cow's milk proteins. Moreover, enhanced immune responsiveness to dietary proteins has been noted in patients with newly diagnosed IDDM (Karjaleinen *et al.* 1992). Animal models that are being used to help elucidate the pathogenesis of this disease are BB rats and NOD mice spontaneously developing diabetes (Rose and Mackay, 2000; Bach, 2002; Shoenfeld *et al.*, 2002).

It was shown that in these animal models, autoimmune diabetes could be inhibited by dietary manipulations and induction of oral tolerance by feeding autoantigens. Our findings demonstrating that a gluten-free diet in NOD mice prevents onset of diabetes suggest the role of the gut in induction of diabetes (Funda *et al.*, 1999). NOD mice were derived in our laboratory into a GF condition, and the effect of microflora components is being assessed. It is interesting that in our preliminary experiments, it seems that autoimmune diabetes is developing also in GF NOD mice.

Atherosclerosis

Recently, atherosclerosis was shown to begin as an inflammatory disease affecting endothelial cells and other compartments of arterial wall. This inflammatory reaction, characterized by accumulation of macrophages and T and B lymphocytes, is accompanied by fatty depositions on the walls of the blood vessels, leading to their occlusion. During the past several years, various bacteria have been implicated in the etiology of this disease (Bachmaier *et al.*, 2000; Kiechl *et al.*, 2001): the intracellular bacterium *Chlamydia pneumoniae*, *H. pylori*, and various oral bacteria, suggested also as causative agents of periodontitis (*P. gingivalis*, *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans*).

Increased levels of antibodies to Hsp 60 in the population of the people exhibiting atherosclerotic changes in their vessels and experiments on animal models indicate that infection leading to increased production of Hsp 60 antibodies could, by similarity with human Hsp 60 expressed under stress in bifurcation of the vessels, trigger the inflammatory disease (Bachmaier *et al.*, 2000; Kiechl *et al.*, 2001; Wilson *et al.*, 2002). Thus far, one mouse model prone to developing atherosclerosis, the apolipoprotein (apo) E $-/-$ mouse, has been derived and maintained in GF conditions (Wright *et al.*, 2000). These GF mice show no detectable differences from their CNV-reared counterparts in the time course of development or severity of the atherosclerotic symptoms.

Allergy

A sudden increase in allergies occurring in recent years and a continuation of this trend in economically developed countries have triggered interest in factors in the external environment. The results of recent epidemiological studies showed that their incidence today is almost three times higher than it was in 1970. This increase, which concerns mainly economically well-developed countries, was not found in the former socialist countries (Russia, Romania, Bulgaria). A search for an explanation for this trend resulted in a hypothetical statement that exaggerated hygienic conditions in developed countries decreased the quantity of natural infectious stimuli from the external environment, disturbing well-balanced development of subpopulations of T cells (the so-called hygienic hypothesis about the increase in allergies).

In early infancy the immune system and functions are not yet fully developed. At birth, Th2 cells are predominantly active, but this changes in favor of Th1 cells, providing a normal course of early postnatal development. Because the microorganisms colonizing the intestinal tract of newborn infants are the first microbial stimuli to which they are exposed soon after birth, it stands to reason that intestinal flora may be one of the principal candidates for providing an explanation for this phenomenon. Recent microbiological analyses pointed out differences in the composition of intestinal microflora between children from highly developed and underdeveloped countries. The former are born under controlled conditions in nursing homes under maximal care and observance of all hygienic measures. Consequently, the spectrum of microbes of their intestinal tract is much more limited than that of children in less-developed countries (Bjorksten *et al.*, 2001; Tlaskalova-Hogenova *et al.*, 2002; Kaliomaki and Isolauri, 2003). Unfortunately, there is still little understanding of the role played by intestinal lymphatic tissue and mucosal immunity in these processes, and there is a paucity of animal models for food allergies that could be manipulated to assess the effects of the "normal" intestinal microbiota.

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