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MUTUALISM BETWEEN THE MUCOSAL IMMUNE SYSTEM AND COMMENSAL INTESTINAL BACTERIA (PART I)

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SUMMARY

Members of the ordinarily benign, ‘normal’ intestinal microbiota stimulate the development and maintenance of the adaptive and innate gut immune system. Comparisons of antigen-free (AF), germ-free (GF), and conventionally-reared (CNV) mice indicate that absolute cell numbers, cell subset distributions, and activation states of both mucosal and systemic elements of immunity can be differentially affected by particular gut microbes. Colonisation by Gram-positive segmented filamentous bacteria (SFB) vs. Gram-negative *Morganella morganii* will be used to illustrate effects. A further type of experiment using such gnotobiotic mice is aimed at determining whether continuous gut colonisation by either SFB or the pair of Gram-negative bacteria (Schaedler’s *M. morganii* plus *E. coli*) is required to maintain the steady state level of ‘natural’ and specific IgA production in the gut. A dietary ‘shift down’ to an AF diet eliminates SFB but not the two Gram-negative microbes from the gut without affecting IgA production over 4-7 weeks in either case. Further, a role for gut microbes in driving the development of IgA blasts in μMT gene segment (-/-) mice will be supported.

Use of comparisons of GF vs. gnotobiotic mice will be presented illustrating roles for gut microbes in: (a) contributing to ‘homeostatic’ and ‘greater than homeostatic’ proliferation of T cells; and (b) the role of particular gut microbes in upregulating expression of various products of gut epithelial cells (ECs). In the latter, gene-profiling analyses were used to analyze expression of RELM-β (goblet cells) and Reg III (autocrine growth factor, ECs) stimulated by particular gut microbes. Finally, the striking finding of pauci-dispersity of gut IgA plasma cells in even CNV mice will be considered. The roles and mechanisms by which microbial TI-1 and TI-2 Ags may stimulate a pronounced oligoclonality of gut IgA cells expressing germ-line or near germ-line Ig V-genes amongst the overall population of IgA blasts will be discussed and model systems using chronic colonisation of mucosal surfaces by microbes expressing such TI Ags will be presented.

GENERAL HISTORIC INTRODUCTION

'Natural', benign microbial colonisers of the gut have long been thought to have a role in stimulating the normal development of both innate and adaptive elements of the mucosal immune system and in maintaining its 'natural state' of activation. The main support for this role has come from comparing the numbers and phenotypic/functional properties of various subsets of cells of the mucosal immune system – B cells, T cells, NK cells, etc. – found in the intestine of germ-free (GF) or antigen-free (AF) hosts vs. those reared conventionally (CNV) (*Crabbe* et al., 1970; *Crandall* et al., 1967; *Cebra* et al., 2005; *Bos* et al., 2003). The general finding is that many fewer of such cells – for instance IgA-blasts, α/β TCR^+ T cells, cytotoxic NK cells, CD45RBlow/$CD4^+$ T cells, etc. – are found in the germinal centres (GCs), lamina propria, or intraepithelial leukocyte (IEL) spaces of the guts of GF vs. CNV mice. Our group and others have compared the gut mucosal system of GF mice vs. mice monoassociated with a variety of ordinarily benign, non-invasive, single species of bacteria (*Shroff* et al., 1995; *Talham* et al., 1999; *Umesaki* et al., 1995). Generally, all such species of commensal microbes stimulate the development of GC reactions (GCRs), the production of IgA blasts which accumulate in the lamina propria and secrete both 'natural (total)' and specific IgA. Individual species of normal gut colonisers seem to differ in the maximal amount of total IgA they induce and the relative amount of this that can be shown to be specific vs. the microbial Ags of the inducer (*Bos* et al., 2001). All seem to induce a waxing and long term waning of the secreted IgA and a much more rapid development and decline of GCRs. An example of such a perturbation of the humoral response is given by segmented filamentous bacteria (SFB, related to the Clostridia), which becomes a major gut coloniser of the distal ileum of CNV as well as GF mice between weaning and puberty. Colonisation of formerly GF weanlings results in transient GCRs, the populating of lamina propria by IgA plasma blasts, and levels of IgA production about 50-70% of that seen normally in CNV mice. Only about 1% of this IgA can be shown to be specific for the SFB (*Talham* et al., 1999; *Jiang* et al., 2001). Colonisation of GF mice with SFB also shifts the prevalent ratio of $CD4^+$ subsets from about 65:35 CD45RB-high:CD45RB-low cells to the reverse ratio of 35:65. The specificities of these 'activated' $CD4^+$ T cells for microbial Ags has not been shown, as is the case for most activated gut T cells presumably induced *in vivo* by microbial products. We have used neonatal/young adult GF mice, monoassociated with SFB, to address a number of follow-up questions concerning host mucosal immune system/microbial coloniser interactions:

1) Can mice monoassociated with SFB for long periods develop a new round of GCRs in the gut upon super-colonisation with a different commensal microbe? After about 100 days following monoassociation with SFB, mice were super-colonised with *Morganella morganii* (Gram-negative, facultative anaerobic rod). GCRs again developed, as did a novel, specific IgA Ab response vs. *M. morganii*. Little change in overall production of total IgA in the gut was detected although the maximal, specific responses to *M. morganii* were about 20x greater than found vs. SFB. Thus, chronic GCRs, observed in CNV mice, are likely due to overlapping, following continuous exposure of the gut to novel microbial Ags (*Talham* et al., 1999);
2) Although the GCRs waxed and waned following monoassociation of

formerly GF mice with a particular microbe, the increased level of lamina propria plasma cells and the output of 'natural' and specific IgA in the gut changed only gradually, if at all, even over periods greater than 100 days post-colonisation. The more rapid decline of GCRs could be attributed to the 'blocking' or 'shielding' of B cell follicles from microbial Ags by the effective production of specific IgA Abs. However, the productive IgA plasma blasts, both specific and 'natural', persist for much longer periods. This finding raises the possibility that long-term persistence of the bacteria in the gut can provide the necessary, continuing stimulation of the previously initiated specific and 'natural' IgA production. In order to test this possibility we performed a dietary 'shift down'. This approach was based on our observations that SFB could not colonise the gut of mice maintained on a chemically defined (CD) diet (*Pleasants* et al., 1986), while a pair of facultative anaerobes, *E. coli* (Schaedler) and *M. morganii*, could. After colonisation of GF mice raised on regular mouse chow (conventional, CNV), groups of colonised mice were periodically shifted to the CD diet (so-called AF-diet) and analyzed for gut bacterial counts and gut IgA production in comparison with littermates that continued on CNV diet. After colonisation with either type of microbe, total IgA production by gut fragments rises over 3-4 weeks and remains rather constant up to 7 weeks. The total IgA output stimulated by the two Gram-negative microbes is about half that given with SFB, and specific IgA Ab follows the same time course except that the specific Abs given by the pair of Gram-negative enteric rods was about 40x higher than that in SFB colonised mice (absolute levels) or 20x higher on a basis relative to total IgA produced. Upon 'shifting down' to the CD, the faecal level of *E. coli* dropped by about one order of magnitude (E+10 to E+09) while the level of *M. morganii* increased by about 1.0 to 1.5 orders of magnitude to E+09. By the end of the experiment both mice on CD and CNV diets had rather balanced bacterial populations of 10^9/g of each species. The SFB quickly dropped to non-detectable over the 2-3 days following the dietary shift, although mice maintained on the CNV diet retained a near constant level of faecal SFB. In both groups of mice colonised with a particular organism(s), the overall level of total IgA and specific Ab remained unperturbed over the period of dietary shift and for four weeks beyond, even though the SFB were effectively eliminated while the coliform were not. Thus, the maintenance of levels of total IgA and specific IgA anti-microbial Ags was not affected by the dietary shift, whether the gut bacteria was effectively eliminated or not. These observations suggest that continuous, overt colonisation is not necessary to maintain rather long-lived production of IgA in the gut, although Ag-retention on dendritic cells, such as FDC or IDC, cannot be excluded as a source of continuous stimulation. It now seems relevant to revisit the half-lives and turnover of IgA plasmablasts in the gut;

3) Finally, we have addressed the issue of whether mucosal Abs, obtained by neonates during suckling, could affect the numbers and whereabouts of particular gut commensal microbes. Since SFB appears in the small intestine only after weaning and rises rapidly to become one of the most abundant enteric bacteria there before disappearing at 8-12 weeks, we suspect a role for mucosal IgA and/or IgM in these changes. Pups born of

immunodeficient (SCID) mothers show a precocious rise in SFB in the terminal ileum and its persistence there to adulthood compared with offspring of immunocompetent mothers (*Jiang* et al., 2001). By conducting swaps of pups at birth which were either immunocompetent or SCID to nurse mothers that were either immunocompetent or SCID, we demonstrated that suckling on immunocompetent nurse mothers forestalled the small gut colonisation compared with pups having SCID nurse mothers. The genotype of the pups: scid/scid vs. scid/+ only had an effect after weaning to determine the persistence or not of SFB in the ileum. The persistence of the SFB was only evident in pups who themselves were (scid/scid). Recently, mice with the AID k.o. have been used to argue that these effects of nursing on SFB colonisation depended on Ig isotype switching and/or the point mutational process leading to affinity maturation (*Suzuki* et al., 2004). A likely target for a specific Ab effect could be those 'hold-fast' structures that anchor the SFBs to the brush border of ileal epithelial cells, allowing their persistence at this turbulent site.

PARTICULAR EXAMPLES OF THE *IN VIVO* EFFECTS OF COMMENSAL BACTERIA/HOST MUCOSAL IMMUNE SYSTEM INTERACTIONS

Some common approaches to implicating members of the 'normal' murine microbiota in initiating or maintaining 'normal' cellular responses evident *in vivo*:

1. Compare the particular response in CNV vs. GF mice or in gnotobiotic (defined flora, DF) vs. specific pathogen free (SPF) mice;
2. If the response is qualitatively or quantitatively different between the pairs of mice being compared, attempt to mono- or oligoassociate GF mice with particular, defined members of the 'normal' microbiota (make DF or gnotobiotic mice) to try to mimic the condition of full 'natural' colonisation and its effect on the particular phenomenon of interest;
3. If one or several defined microbial species can initiate the effect, attempt to identify the microbial Ags/ligands and their target mammalian cells/receptors that are involved in the initiation and/or maintenance of the phenomenon;
4. Try to establish any 'cross-talk; that may lead to indirect effects on cell types not directly interactive with the microbial product;
5. Try to understand the operative mechanisms and the consequences of the host cell responses for normal homeostasis or resistance/protection vs. pathogens. For instance, if a mammalian cellular product is induced by a microbial stimulus, does it act in an autocrine manner, does it target other host cells and/or does it have anti-microbial effects?

Recently, attempts to genetically 'knock out' particular, potential microbial agonists or host cell receptors/products have culminated such studies as outlined above. However, often these have been frustrated by redundancies of genes for relevant products and common signalling pathways.

The status of four projects carried out along the lines summarised above, which have progressed to varying levels of understanding, will be presented. It is postulated that in each case, microbial products either induce, amplify, or contribute to the maintenance of particular mammalian, cellular responses. It is ex-

pected that the description of these phenomena will stimulate much discussion and further work.

A. In 2001 a paper by *Macpherson* et al. (2001) appeared which described a 'leakiness' of IgA production in CNV μMT (-/-) mice. These mice have a homozygous deletion of the gene segment encoding the transmembrane portion of the μ-heavy chain components of their BCRs. Thus their B cell development was blocked at the pre B cell stage. Nevertheless, at about 5 weeks of age most but not all of these pups expressed about 1-50% of normal serum IgA levels. These mice carried the so far undefined SPF flora of the Zurich mouse colony. Our own studies utilised several lots of μMT breeding stock from the Jackson Labs SPF colony, directly obtained from the founder colony in Köln (*Kitamura* et al., 1991). Our CNV μMT mice and W/T littermates were maintained in Trexler isolators using those sterile procedures appropriate for GF mice. Our findings agreed qualitatively but not temporally or quantitatively with those of the Macpherson group. We found only sporadic expression of serum IgA in a small fraction of mice up to 7 months of life and the concentrations were usually <1% of normal, W/T mouse levels. After one year of age, more mice (about 50%) were serum positive for IgA but again at around >1% of normal levels. Such low and sporadic expression of IgA suggested that successful transit of B cells through the pre-B cell stage was a rare event in these mutant mice and may be reflected in the mono- or oligo-clonality of their IgA blasts. Thus we tested cell samples from different regions of the gut for cells expressing the same CDR3 sequences (clonal relatives) and the occurrence of point mutations in the Vα region of IgA using PCR cloning and DNA sequencing. The surprising result from analyses performed on samples from one 13 month old mouse was that most relevant, cloned cDNA sequences from its productive, recombined Vα genes were clonally related and differed by only 1-2 VH mutations from the GL sequence. This GL sequence, from our previous studies, is expressed in IgA antibodies reactive with α1_6 dextran, a microbial product. Of course, we must develop these observations further, but our surmise is that active VDJ gene, associated with the truncated Cμ gene, can be translocated to the usual switch recombination region 5' to the intact Cα gene, even in the absence of external stimulation via BCRs. Such rare B cells, now expressing membrane-associated IgA as BCR, may be 'rescued' specifically by gut microbial products and be expanded to form rather large clones. Thus, appreciable rescue events would depend on a rather large repertoire of microbial ligand and the temporal and quantitative difference between our findings and those of the Zurich group could well be due to a very different, more diverse microbiota in the mice used by the latter. Further, one might expect that those specificities of B cells we detect as clonal products may signify those very common microbial ligand - matched to GF gene VJH/VL pairs - that specifically stimulate very early TI-responses. Finally, an attempt to enhance the detection of 'leakiness' in our mice by superimposing *Trichuris muris* infection on their normal SPF flora was unsuccessful.

B. Transfer of congenic, unfractionated or naïve (CD44low T cells), labelled with a vital fluorescent dye (CFSE),

into 6 Gy irradiated hosts results in a rather slow outgrowth of T cells. Reactivity with self-peptide ligands was implicated as a stimulus for such expansion, called 'homeostatic proliferation' (*Jameson*, 2002; *Moses* et al., 2003; *Kieper* et al., 2004). More recently, we found that such cell transfers into non-irradiated, immunoincompetent hosts [H-2^b RAG (-/-) or H-2^d C.B17 SCID mice] led to a much more extensive proliferation of both $CD4^+$ and $CD8^+$ donor T cells (*Kieper* et al., 2005). Many of these, when analyzed after proliferation and loss of detectable CFSE, were CD44high, CD25positive, and expressed TNF-α, γ-interferon, or both. Further, we found that transfer of CFSE-labelled T cells into GF SCID mice compared with CNV SCID hosts resulted in a marked diminution of expansion to 'homeostatic levels'. Thus, it seems likely that gut microbial products may markedly augment the stimulus by 'self' peptides for cells to proliferate *in vivo*. So far, GF SCID mice monoassociated with one of several common commensal bacteria provide a rather small increment of 'extra' expansion of T cells. Possibly, the marked expansion we observed in CNV SCID mice reflects the superpositioning of many specific T cell responses to gut microbial Ags? Obviously, we should use a variety of oligoassociated SCID mice (*i.e.*, DF) to try to detected an additive effect in stimulating T cells outgrowth *in vivo*.

It has become popular to search for tissue-specific products whose expression may be upregulated by gut microbes using first Northern blotting developed with cDNA probes and then gene expression profiling via cDNA arrays on microchips. We have studied two such products expressed in mouse large intestine and apparently up-regulated by colonisation of formerly GF mice with whole SPF microbiota or particular gut commensal microbes.

C. Using cDNA probes, Northern blotting, and a polyclonal anti-peptide antiserum, we have shown the increased presence of RELM-β (resisten-like molecule) (*Steppan* et al., 2001) in the secretory granules of goblet cells from the large intestine upon colonising formerly GF BALB (immunocompetent) or C.B17 SCID mice with SPF microbiota (carried by Jackson Lab. mice) (*He* et al., 2003).

The prodigious amount of product, RELM-β, can be easily extracted from faecal pellets or isolated from colonic washes. The rise in expression of RELM-β can easily be detected within 24 hours of exposure of GF immunocompetent or SCID mice to SFB microbes. Thus, an adaptive immune system is not required to participate in this up-regulation of RELM-β expression induced by colonisation with the Jackson SPF microbiota. At the level of RELM-β mRNA expression, about a 7-8 fold increase is also noted. Of interest and a cause for some chagrin, is that monoassociation of GF BALB mice with SFB, *Helicobacter muridarum*, *E. coli*, or oligoassociation with the 'altered Schaedler's flora' fail to result in any increase in expression of RELB-β. Thus, it would appear that identification of microbial agonists might be stymied. However, it has recently been shown that supercolonisation of CNV mice with embryonated eggs of helminths, such as *Trichuris muris*, result in the induction of very high levels of RELM-β, such as 40x the levels of expression of mRNA found in CNV mice bearing the complete SPF microbiota. The fate of these recently hatched worms is expulsion in 10-18 days, coinciding with a marked Th2 T cell response. Along with the adaptive

immune system, it seems possible that the copious RELM-β produced may also play a role in expulsion by accumulating in the bacillary pores of the worm (*Artis* et al., 2004). However, it is relevant to note that SCID mice, which also somewhat more modestly up-regulate RELM-β expression when colonised with *T. muris*, develop a chronic helminth infection.

D. Using gene expression profiling via cDNA arrays on microchips we compared fold induction of mRNA from GF, monoassociated, oligoassociated, and CNV mice that were either immunocompetent BALB or SCID (C.B17). We found a set of genes, RegIIIβ and RegIIIγ, that were upregulated in the colon about 9-11 fold in SFB monoassociated and in DF (altered Schaedler's flora) colonised SCID mice compared with expression in colons of GF SCID mice (*Keilbaugh* et al., 2005). The RegIII gene family is related to human pancreatitis associated protein (PAP), which induces cellular proliferation and inhibits apoptosis (*Clark* et al., 2000). The intestinal versions of this gene family may be autocrine growth factors made by gut epithelial cells. Because γ-interferon, and several other genes upregulated by γ-interferon such as IRG47, Rab6-kinesin like, UPase, guanylate nucleotide binding protein, were also up-regulated in the colons (2-6 fold) of the same group of colonised mice, we initially supposed that colonisation of SCID mice led to activation of components of the innate immune response – for instance NK cells which produced γ-interferon – and the subsequent up regulation of the RegIII genes occurred secondarily. Thus, we assessed the effect of monoassociating GF mice with *E. coli* (Schaedler) on expression of mRNA transcribed from RegIII genes and γ–interferon gene in the colon, since we had found that colonisation of GF mice with *E. coli* was particularly effective at activating NK cells in the IEL compartment. Our findings were:

1) that increases in relative expression of all these genes was much greater in formerly GF SCID mice vs. GF immunocompetent mice – suggesting an antagonistic effect of the specific, adaptive immune systems in immunocompetent mice;
2) that the up-regulation of γ-interferon expression was temporally distinct from that of the RegIII genes; and
3) that the natural mouse *E. coli* was more effective than other microbes used to colonise GF SCID mice in up-regulating RegIII expression (*Keilbaugh* et al., 2005).

Thus, it seemed that the host response to colonisation by *E. coli* was based on its innate immune system, but we are unclear about the mechanisms of action of its microbial products. We further pursued the possibility that activated NK cells would play an intermediate role in the increased expression of RegIII genes. Although we found an increase in activated NK cells expressing γ-interferon in the IEL spaces of *E. coli* colonised SCID mice, pre-treatment of these mice with MAb anti-γ-interferon did not depress expression of RegIII genes but did markedly decrease expression of the colonic γ-interferon gene and of the γ-interferon dependent UPase gene (*Keilbaugh* et al., 2005). Recently, some further relevant findings have been reported by Lora Hooper and colleagues at the ASM Symposium: Beneficial Bacteria (*Cash* et al., 2005). These included:

1) that RegIIIβ and γ genes are also up-regulated in the small intestine of CNV compared with GF mice and Paneth cells seemed to be

major producers of protein product;

2) these RegIII proteins appear to be animal lectins, reactive with peptidoglycan (especially its N-acetyl glucosamine component); and
3) these proteins appear to selectively inhibit clonal outgrowth of Gram-positive but not Gram-negative bacteria, using *in vitro* culture procedures.

It remains to be determined whether such anti-bacterial action is manifest *in vivo* and whether any particular gut commensal bacteria of the SPF-mixture can induce expression of the RegIII proteins in small intestinal Paneth cells.

POSSIBLE ROLES FOR COMMENSAL, MICROBIAL ANTIGENS/LIGANDS IN EXPANDING, SHAPING, AND MAINTAINING THE 'NATURAL' MUCOSAL IgA ANTIBODY REPERTOIRE

Relative to the systemic 'natural' and 'specific' antibody repertoire, the IgA antibodies made by the abundant IgA plasmablasts in the gut show striking differences. For instance, using CDR3 length spectrotype analyses of recirculating, follicular, splenic IgM^+ B cells, their productive Vμ genes show a symmetric distribution of CDR3 lengths. However, a similar set of analyses of productive, intestinal IgA-blasts show an oligodisperse distribution of CDR3 lengths (*Stoel* et al., 2005). This observation is the same whether monoassociated SCID recipients of B cells are analyzed or whether CNV immunocompetent mice of several common strains are examined: BALB/c, C57BL/6, and CBA. Likewise the findings are the same whether CBA (*xid/xid*) mice, deficient in B1 cells, or wooley mice, deficient in B2 cells are similarly analyzed. These rather qualitative observations were supported by DNA-sequencing of CDR3 regions and then VH-regions from productive IgA-plasmablasts. Surprisingly, out of 15-20 sequences examined from various types of mice, two or more likely clonal relatives were found, based on identical V/D/J junctional sequences. The relationship was verified by finding that these usually differed in Vα-sequences and/or were GL or near-GL, with unrelated point mutations that were not clustered in CDR-regions and did not show significant increase in R/S type mutations (*Stoel* et al., 2005).

It seemed to us that if microbial, polyclonal stimuli accounted for the development of these B cells likely making 'natural' IgA antibodies, then polydisperse B blasts would be expected. Perhaps either B1- and/or B2-cells, stimulated specifically by TI-1 and/or TI-2 Ags via their BCRs, allow for an oligodisperse population of IgA-blasts making 'cross-reactive' but <u>not</u> polyreactive natural antibodies. Perhaps such B blasts, with randomly recombined V/D/J segments but without appreciable N-additions or point mutations in Vα regions, are selected in the gut by relatively few TI antigens?

We have tried to first evaluate this possibility by assessing whether either or both B1- and B2- cells making IgA in the mucosa show any evidence of having been selectively and specifically stimulated by microbial TI Ags, based at least in part on their BCR? Particularly:

1. Can B1-cells be selectively and specifically stimulated by a TI-1 Ag – such as LPS bearing a particular antigenic determinant – via their BCRs and go on to secrete IgA Abs?

   In order to approach this issue in a way that permitted identification of B1- or B2- cells *ex vivo*, we created

Igh-allotype distinctive, congenic chimaeric mice by transferring B1- or B2-cells from different sources (C.B20, Ighb vs. BALB/c, Igha) into formerly GF SCID (C.B17) mice. In order to provide any necessary, bystander T-cell help we made reciprocal mixtures of purified B1- cells from peritoneal cavity cells (PeC) of one Igh allotype and added these to the congenic, B1-cell depleted Pec (including B2-cells, T cells, APCs, etc.) of the other Igh allotype and then transferred these mixtures. In order to distinguish the effects of possible BCR-specific determinants borne on an LPS from its polyclonal effects, we collaborated with the Weiser group, who prepared two genetically-manipulated strains of *Haemophilus influenzae*: One that constitutively added the phosphocholine prosthetic group (PC) to its LPS and the other that lacked the necessary, functional enzymes to attach PC to the same LPS moiety (*Lysenko* et al., 2000). Thus we could monoassociate B-cell reconstituted SCID mice with one or another of the *H. influenza* strains. We found (*Lysenko* et al., 2000):

a) That neither the PC^+ not the PC^- strain chronically colonised the intestine of SCID mice but that each colonised the upper respiratory tracts about equally for up to six months;
b) At two weeks after stable colonisation of 8-10 weeks old GF SCID mice with either the PC^+ or the PC^- strains of *H. influenzae*, lymphoid cells were transferred. At 10 weeks after cell transfer, mice monoassociated with either the PC^+ or PC^- bacteria showed 'natural' or 'total' IgA production by their NALT and respiratory lymphoid tissue (RALT) *ex vivo*. Those mice colonised with the PC^+ strain showed roughly 10x higher levels of total IgA production;
c) Most of this 'natural' IgA production was made by the B1-cells in the Igh-allotype chimeras – about 10x as much as could be attributed to B2-cells – if the recipients had been colonised with the PC^+ strain of *Haemophilus*. If the PC^- strain was used for colonisation, B1- cells showed almost no contribution to the total IgA output, while the B2-cells produced about the same amounts of 'natural' IgA as did their counterparts from PC^+ colonised mice;
d) Examination of the same tissue fragment culture supernates as used above to detect 'total' IgA for detection of specific anti-PC antibodies showed that only mice colonised with PC^+ *Haemophilus* made detectable specific Abs, and B1-cells contributed about 30x more of these than did B2-cells. Much of the 'total' IgA made by B1-cells could be accounted for by the anti-PC component;
e) Perhaps surprisingly, almost all the B1-cell derived anti-PC was positive for the T15-idiotype, using MAb A.B1 for detection (*Benedict* and *Kearney*, 1999). This MAb specifically reacts with Abs expressing the VH/VL pair of germ-line (GL) or near GL-gene associated with the dominant type of anti-PC made by these mice;
f) Finally, the extent of colonisation of upper respiratory tract remained about the same for both PC^+ and PC^- strains of bacteria, whether or not the hosts were making anti-PC in RALT or NALT.

Thus, the LPS of the PC^+ vs. PC^- strains did not contribute detectably to the anti-PC response by polyclonal stimulation. Instead, the PC-prosthetic group on PC-LPS seemed to

selectively and specifically activate B-cells, at least in part, via their BCRs. Of course, LPS interactions with the CD14/TLR4 complex may have played a co-stimulatory role *in vivo*.

2. Can either B2-and/or B1-cells be selectively and specifically stimulated *in vivo* by microbial TI-2 Ags based on their BCR and then go on to generate IgA Ab secreting cells?

   In order to approach this issue with any likelihood for quantitatively detecting IgA Abs specific for microbial TI-2 determinants we believe it necessary to:

   a) Identify enteric microbes that carry TI-2 determinants, known or shown to react with IgA MAbs that expressed GL- or near GL-V genes;
   b) Show that such microbes could chronically colonise the guts of GF mice;
   c) Show that such microbial colonisers could stimulate an appropriate and detectable, specific IgA Ab response *in vivo*. Of course, such a finding would be followed up by a molecular genetic analysis to discern oligoclonality and assess the pattern of point mutations among any clonal relatives detected (see above). Such a response might implicate chronic bacterial TI-2 Ag stimulation of the host's B1- and/or B2-cell populations in establishing the partly oligoclonal occurrence of 'natural' IgA producing cells in the guts of mice not deliberately challenged by enteric Ags.

   The earlier attempts to establish Ag-specificities for the 'spontaneous' IgA plasmacytomas that arose in BALB/c mice as well as our own evaluation of a set of eight MAb hybridomas (*Bos* et al., 1996) have indicated that many of these express GL- or near GL-V genes and may react with determinants found on microbial poly- or oligosaccharides (TI-2 Ags). Examples of such specificities include α1-6 dextrans, β2-1 fructosans and PC-borne by teichoic acids. Indeed MAbs from plasmacytomas and hybridomas are available for testing such specificities borne by particular microbes and Ags suitable for ELISA or RIA assays are available for evaluating gut IgA responses made by mice chronically colonised by candidate microbes that might express IgA Abs of such specificities.

Thus far, our results have been meager in attempts to identify suitable colonising organisms. The experimental protocol discussed above needs to begin with GF or gnotobiotic mice in an attempt to exaggerate any response to TI-2 Ags. We have found an encouraging approach to search for commensal gut bacteria that may bear determinants of the above listed TI-2 Ags. We have used a set of defined flora (DF: *Lactobacillus salivarus*, *M. morganii*, SFB, and *Bacteriodes distasonis*) to colonise GF IgA (-/-) mice (H-2b haplotype). After two weeks bacteria are harvested from gut, presumably expressing *in vivo* characteristics, and stained with PI plus MAb reactive with β2-1 fructosyl or PC-determinant. Because the host mice are IgA (-/-), they do not show the usual 'endogenous' coating of nearly all gut bacteria with their own IgA. We find that MAb ABPC47, reactive with β-fructosyl, stains 16% and MOPC603 or TEPC15 MAbs, reactive with PC, stain 2.7% and 12% of the live bacteria respectively. Thus, it might be possible to relate the expression of these TI-2 determinants to particular bacterial species.

However, monoassociation of GF mice with particular single bacterial species – *M. morganii*, Schaedler's *E. coli*, *L. salivarus*, SFB – has failed to result in IgA Abs detectable above the general rise in 'natural' IgA and reactive with either PC^-, β2-1 fructosyl or α1-6 dextran. Possibly we have not so far identi-

fied an example of a gut bacterial species that does specifically stimulate BCRs related to those commonly found to be expressed by 'spontaneously' or 'randomly' arising plasmacytomas, hybridomas, or 'leaky' B cells in μMT mice (see above). Possibly, one should try gut colonisation with *Aerobacter levanicum*, a free living bacterium that ordinarily does not colonise animals, since it is the source of the prototypic fructosan Ag that has been delivered parenterally to mice to study the stimulation of B cells expressing anti-fructosyl antibodies (*Boswell* and *Stein*, 1996).

We hope that our many imperfect and incomplete *in vivo* experiments seeking to better understand reactivity of microbial TI Ags with mammalian lymphoid cells will stimulate further, more sophisticated attempts to understand the relationships between 'natural' and 'adaptive' immune responses. We especially hope that we have been persuasive of the benefits of using GF and gnotobiotic mice compared with different conventionally reared mice such as 'SPF' with imperfectly defined but partially perturbed gut microbiota.

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