Old Herborn University Seminar Monograph 7:
Immune system and microflora.
Editors: Peter J. Heidt, Volker Rusch, and Dirk van der Waaij.
Herborn Litterae, Herborn-Dill, Germany: 67-84 (2003).

THE INTERACTION OF THE MUCOSAL IMMUNE SYSTEM OF THE GALT WITH INDIGENOUS BACTERIA AND ENTERIC VIRUSES

JOHN J. CEBRA[1,2], NICOLAAS A. BOS[2], ETHEL R. CEBRA[1,2], DAVID R. KRAMER[1], FRANS G. M. KROESE[2], KHUSHROO E. SHROFF[1], and ROBERTA D. SHAHIN[1]

[1]Department of Biology, University of Pennsylvania, Philadelphia PA, USA, and
[2]Department of Histology and Cell Biology, Immunology Section, University of Groningen, Groningen, The Netherlands

SUMMARY

Our observations of gnotobiotic and antigen-free adult mice and of conventionally-reared neonatal mice following their colonisation with microbial flora or infection with enteric viruses suggest that these latter mucosal antigenic stimuli play a major role in the development and maintenance of the normal elements of the mucosal immune system in Peyer's patches, intestinal lamina propria, and the intra-epithelial leukocyte compartment. We have developed a number of novel assay procedures to evaluate a gut mucosal immune response including: (1) detection of germinal centre reactions in Peyer's patches by fluorescence-activated cell sorting; (2) tissue fragment cultures for Peyer's patches and small intestine to detect secreted specific and total antibodies; (3) single or clonal B cell microcultures to assess frequencies and isotype potential of specific B cells; and (4) detection of endogenous coating of gut bacteria by IgA antibodies by the host. Using these methods we have shown that initial stimulation of the gut mucosal immune system results in transient germinal centre reactions in Peyer's patches and sufficient secretory IgA antibody to shield or attenuate subsequent or continued gut immune responses. Maternal antibodies, passively acquired by suckling, can both protect neonates and forestall their active mucosal responses to both intestinal commensal microbes and to pathogenic enteric viruses. It appears that acute, novel enteric stimulation - for instance with enteric viruses or cholera toxin - can overcome the hypo-responsiveness to naturally occurring 'bystander' antigens and reactivate mucosal immune responses to them. Finally, although the B1 B cell subset, including specificities cross-reactive with bacterial antigens, seems an attractive complement to the B2 B cells, acutely primed in the Peyer's patches, in populating the gut lamina propria with IgA plasma cells, we have failed to demonstrate a significant contribution for these B1 cells in physiologically normal, immunocompetent mice.

INTRODUCTION

Some years ago our laboratory implicated clusters of lymphoid follicles (Peyer's patches, PP) found in the small intestinal mucosa as major sites for the

development of IgA-committed pre-plasmablasts and for priming of a subsequent IgA 'memory' response (*Craig* and *Cebra*, 1971; *Gearhart* and *Cebra*, 1979; *Fuhrman* and *Cebra*, 1981; *Lebman* et al., 1987). Following effective gut mucosal stimulation by antigens there is an efflux of specific IgA plasmablasts from PP into the efferent lymph and circulation, followed by an accumulation of these cells in exocrine gland interstitia and the lamina propria (LP) of the respiratory and gastrointestinal tracts (*Pierce* and *Gowans*, 1975; *McWilliams* et al., 1975). We still do not know whether the selective lodging of IgA plasmablasts in secretory tissue exhibits any finer preference, for instance a biased accumulation of specific cells in respiratory LP vs. gastro-intestinal LP following exposure of the upper respiratory tract to antigens or vice versa (*Pierce* and *Cray*, 1981). Further, although vascular addressins on the luminal surface of high endothelial venules (HEVs) and corresponding 'homing' receptors on lymphocytes that could account for selective egress of cells from the circulation into LP have been identified, we do not know whether IgA-plasmablasts utilise this recognition system (*Phillips-Quagliata*, 1992). Even if they do, the further bases for their *accumulation* in secretory sites also remains unknown although lymphokines (LKs) abundant at these sites (IL-5, IL-6) may both stimulate their maturation to plasma cells and halt their migration (*Tagochi* et al., 1990).

Together with the development of specific IgA pre-plasmablasts in PP following effective antigenic stimulation of the gut mucosa with cholera toxin there is also a rise in the frequency of specific IgA memory cells (*Fuhrman* and *Cebra*, 1981). Although the dissemination of IgA memory cells to distal lymphoid tissues becomes evident following gut mucosal priming (*Fuhrman* and *Cebra*, 1981), the frequency of these cells remains highest in gut-associated lymphoid tissue (GALT) relative to other sites for long periods thereafter (*Cebra* et al., 1984). The likelihood and magnitude of subsequent secondary mucosal IgA responses positively correlates with the frequency of IgA memory cells in PP following exposure of the gut to antigen (*Fuhrman* and *Cebra*, 1981). We have previously operationally defined IgA-memory cells as those B cells which responded to specific antigens and TH cells in splenic fragment cultures to generate a clone that exclusively secretes IgA antibodies (*Lebman* et al., 1987). These clonal precursor cells from PP are small, non-dividing B cells which are $sIgA^+$, $sIgD^-$, and $sIgM^-$ and *do not* express the characteristic marker of murine germinal centre (GC) B cells, i.e., they do not bind high levels of the lectin, peanut agglutinin and thus they are PNA^{low}.

The sites of generation of IgA memory cells and of IgA pre-plasmablasts within PP have not been unequivocally defined but the chronically present GC are likely locations. There are more $sIgA^+$ B cells in the GC of PP than at any site in any other lymphoid tissue (*Lebman* et al, 1987). Many of these $sIgA^+$ PNA^{high} B cells are dividing (*Lebman* et al, 1987) and most, if not all, have lost at least one copy of their Cμ and Cγl-genes, indicating that they have undergone irreversible switch-recombination (*Weinstein* et al., 1991). Recently, we have shown that GC reactions can be stimulated *de novo* in formerly germfree (GF) mice using oral reovirus infection (*Weinstein* and *Cebra*, 1991). These GC are transient and wax and wane with a time course similar to that observed in draining lymph nodes (LN) after local, parenteral reovirus infection. However, the outcome in terms of Ig isotype-switching is very different at the two sites: There is

Table 1: Isotype patterns expressed by clones from anti-inulin specific B cells taken from formerly germfree mice varying times after colonisation

	Time after Colonisation	Bacterial colonisers	# clones	Clones making (%): A-only	some M	M-only
Sp1[2]	1 week	lactobacilli	10	0	100	70
PP	1 week	"	4	0	100	50
Sp1	1 week	lactobacilli	24	0	92	54
PP	2 week	"	5	20	40	0
Sp1	3 week	lactobacilli	44	0	100	9
PP	3 week	"	18	5	89	0
Sp1	3-18 week	Sch./Convent.[1]	45	29	18	0
MLN	"	"	25	36	20	4
PP	"	"	19	42	11	0
Sp1	52 week	Sch./Convent.[1]	47	9	49	0
MLN	"	"	40	20	55	5
PP	"	"	17	47	12	0

[1] Mice were colonised with Schaedler's commensal bacteria and then conventionalised in a non-SPF animal facility.
[2] Spl = spleen; PP = Peyer's patches; MLN = mesenteric lymph nodes.

prompt expression of Cα germ-line (GL) transcripts in GC of PP, followed by expression of sIgA^{+} B cells and the potential to secrete IgA in culture while LN GC B cells fail to exhibit this preferential switching to IgA expression but rather develop the potential to express IgG isotypes. These findings favour a micro-environmental difference between GALT and peripheral LN sites as accounting for the preferred switching to IgA rather than a difference in physiologic state due to the usual chronic antigenic stimulation of the gut mucosa (*Weinstein* and *Cebra*, 1991). Thus, our system may permit analysis of those micro-environmental factors (DCs, APC, T_H cells, CKs, such as TGFβ, IL-10, etc.) that may favour preferred switch-recombination to IgA expression.

Mice reared under conventional conditions (CNV) harbour an intestinal flora (*Dubos* et al., 1965) and display chronic GC reactions in their PP (*Butcher* et al., 1982). Thus, PP are unlike other lymphoid tissues, which ordinarily contain quiescent B cell follicles without GCs. Analysis of PP from CNV adult mice indicates a frequency of antigenic-specific clonal B cell precursors similar to that found in other lymphoid tissues except that - for some specificities - a much higher proportion are already IgA memory cells. The specificities of precursors accounting for the 'naturally occurring' IgA memory cells include: Anti-phosphocholine (PC), anti-β2→1 fructosyl (Inulin, In), anti-β-galactosyl (β-Gal), etc. (*Potter*, 1971). In fact, most of the identifiable specificities are reactive with bacterial antigens (see *Cebra* et al., 1980). If one examines gnotobiotic mice (germfree, GF) or antigen-free (AF), (*Bos* et al., 1993), one finds for both that PP are vestigial, that their clonal B cell precursors against bacterial determinants are

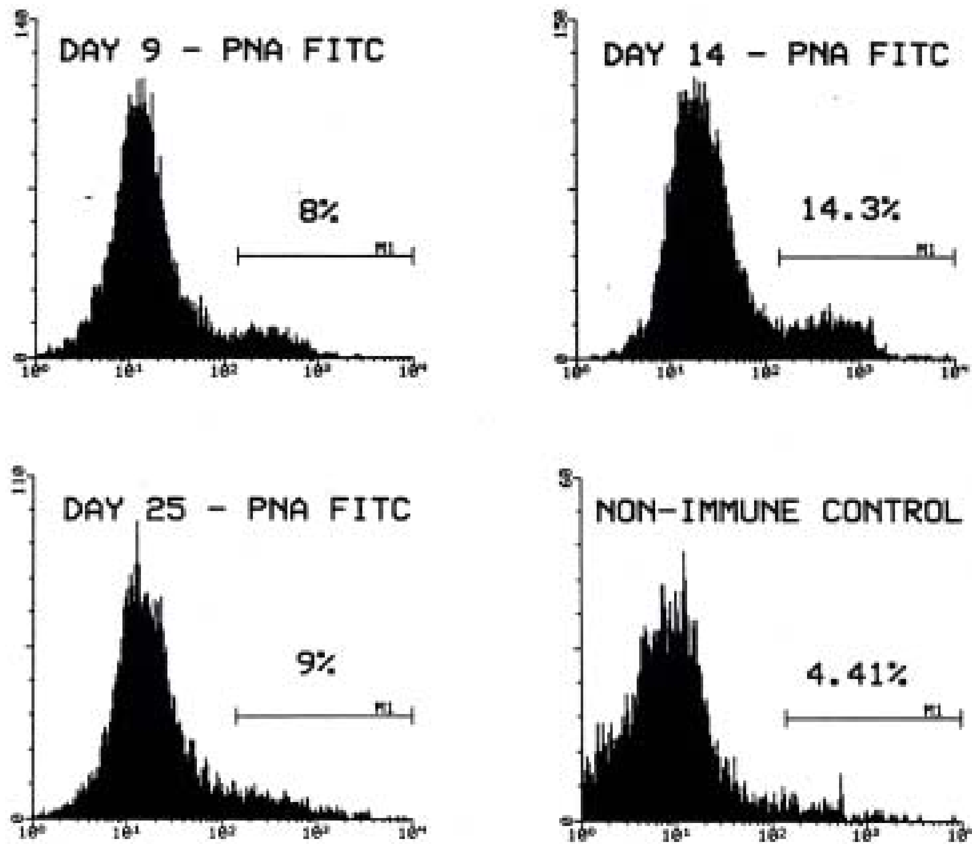

Figure 1: Development of germinal centre reactions in germfree C3H mice colonised with *Morganella morganii*.
(The waxing and waning of the germinal centre reactions in formerly germfree mice after mono-association with *Morganella morganii* by the oral route. Peyer's patch cell suspensions were stained with fluorescein-labelled peanut agglutinin, which preferentially binds to germinal centre cells compared with other cells of the B lineage. Except for the non-colonised control mice, all others harbour high densities of intestinal *M. morganii*).

of much lower frequencies than in CNV mice and few, if any, of these are committed to IgA expression (*Cebra* et al, 1980, 1986).

The AF mice not only lack discernible PP structures, but also show an almost complete lack of IgA and IgM plasma cells in their intestinal lamina propria (*Bos* et al., 1993). These findings suggest a role for the intestinal microflora in the normal development of the humoral mucosal immune system.

Our early studies with GF mice, colonised with the Schaedler 'cocktail' of indigenous bacteria, mostly facultative anaerobes (*Schaedler* et al., 1965), or mono-associated with *Morganella morganii* (*M. morganii*), an occasional commensal of mice (*Potter*, 1971), indicated that:

1. A rise in frequency of B cell clonal precursors occurred both in PP and elsewhere in the first few weeks after colonisation and these were specific for bacterial antigenic determinants;
2. At first most of these precursors were able to generate clones making IgM antibodies and then IgM plus

Table 2: Frequency of anti-inulin and anti-PC clonal precursors in spleens of neonates primed at birth

Donor	# cells analysed ($x10^{-6}$)	Frequency/10^6 cells to: In	PC
3 wks unprimed	1344	1.49	8.88
3 wks In-Hy primed[1]	460	7.07	10.10
1 wk unprimed	80	<0.3	n.d.
1 wk In-Hy primed[1]	80	9.38	n.d.
5 wks unprimed	400	8.91	15.30

[1] 3 day old neonatal mice were primed by intraperitoneal injection of 100 µg of inulin-conjugated to haemocyanin (In-Hy). The clonal precursor frequencies were determined using the splenic fragment assay and Hy-primed recipients and antigenic challenge with either In-Hy or phosphocholine-conjugated Hy (PC-Hy).

other isotypes; eventually precursors appeared with the characteristics of IgA-memory cells, i.e., they generated clones that exclusively expressed IgA antibodies. The latter were always in highest proportion in PP.

Figure 1 summarises some of these studies.

One might consider that neonatal mice exhibit changes in their gut mucosal immune system similar to those observed upon colonising GF mice with enteric bacteria. Neonatal mice are colonised at birth by contamination from their dams. However, they receive maternal IgG antibodies perinatally, first by transplacental passage and later, after birth by transport of suckled IgG antibodies across gut enterocytes. They also accumulate maternal IgA antibodies from milk in their gut lumen. These processes raise questions about whether:

1. Neonates are developmentally competent to mount an active, preferential IgA response in their PPs, and
2. Whether perinatally acquired maternal antibodies can interfere with or modulate this response.

Some years ago we analysed the potential of PP cells from neonatal mice born of CNV parents. The general finding was that the 'spontaneous' development of IgA memory cells specific for antigenic determinants associated with indigenous flora was delayed until 10-12 weeks post partum (*Cebra* et al., 1986; *Shahin* and *Cebra*, 1981). However, in the case of one particular bacterial determinant, β2→1 fructosyl (In), we showed that neonates could be primed by parenteral immunisation with In conjugated to either lipopolysaccharide (In-LPS) or haemocyanin (In-Hy) to exhibit a 'premature' rise of anti-In specific B cells in spleen (*Shahin* and *Cebra*, 1981; see Table 2). Thus, delayed mucosal responsiveness is not due to the absence or paucity of In-reactive B cells. However, these observations still leave unanswered whether the microenvironment of neonatal PPs is underdeveloped in its ability to respond to antigens delivered by the mucosal route or whether maternal antibodies acquired in the milk act to shield the neonatal gut from antigenic stimulation.

Finally, in the past few years, an extra-GALT source of IgA plasma cells that can populate intestinal LP has been proposed (*Kroese* et al., 1989). This is the $CD5^+$ B cell population (B1 cells) that develops perinatally in the liver and

persists throughout life in the peritoneal cavity of some species as an apparently self-renewing population (*Hayakawa* and *Hardy*, 1988). These B1 cells may be stimulated by 'internal' or 'self' antigens and may provide a measure of 'natural' immunity vs. cross-reactive bacterial determinants via their substantial contribution to circulating IgM (*Forster* and *Rajewsky*, 1987; *Lalor* and *Morahan*, 1990; *Riggs* et al., 1990). The actual, physiologic role in mucosal immunity of this potential source of IgA plasma cells, especially in immunocompetent neonatal and adult animals, has yet to be determined and quantified. Further, the possible stimulation of these B1 cells by *exogenous* antigens impinging on the gut mucosa has yet to be evaluated, although preliminary data suggest that cells of this lineage cannot generate GC reactions and therefore may not benefit from the GC microenvironment that facilitates isotype switching and affinity maturation processes (*Linton* et al., 1992).

Special techniques and assays developed to analyse the Rut mucosal immune response

1: *Analysis of the GC reaction in PP of formerly GF mice by fluorescence-activated cell sorting (FACS).*

Our approach was to develop acute, *de novo* GC reactions in PP of GF mice orally infected with reovirus serotype 1 (*Weinstein* and *Cebra*, 1991). Ordinarily, the PP follicles of GF mice do not contain detectable GCs. If the virus-infected mice are kept in isolators under otherwise GF conditions the GC reactions in PP wax and wane while the intestinal virus infection is completely resolved. The GC reactions and antibody expression by B cells from PP of these mice were compared with those from LNs of conventionally reared syngeneic mice inoculated in footpads with infectious virus. GC reactions were initially detected by FACS for the appearance of B cells, which bound high levels of the lectin, peanut agglutinin (PNA^{high}). The PNA^{high} marker has been found to distinguish most GC B cells from others and, in PP of mice, the prevalent phenotype of these is PNA^{high} surface kappa low (SK^{low}). Following acute local antigen stimulation with infectious reovirus, GC B cells appear first at day 6, reach maximum numbers by day 10-12, and decline during the day 14-21 period following infection at both PP and LN sites. The earliest and most persistent GC cells display the phenotype PNA^{high} $s\kappa^{high}$ at both sites, while cells with the PNA^{high} $s\kappa^{low}$ phenotype are only prevalent around the time of maximal GC reaction. While the time courses of the acute GC reaction are similar at the PP and LN sites, the two conspicuously differ in several respects: (1) B blasts appear with the phenotype $sIgA^{+}$ by day 6 and persist through day 19 in PP but not LN and most of these are PNA^{high} $sIgA^{+}$, while a very small component of $sIgG1^{+}$ cells can be detected in LN but not in PP; (2) upon secondary local re-infection with reovirus the GC reactions in PPs and LNs exhibit similar time courses but in the LN they are exaggerated while in the PP they are attenuated compared with the primary reactions; and (3) more $sIgA^{+}$ cells which are PNA^{low} appear in PPs and many more $sIgG^{+}$ cells appear in LNs after re-infection but not *vice versa*. We have found an excellent quantitative correlation between the proportion of PNA^{high} B cells or the appearance of $sIgA^{+}$ or $sIgG1^{+}$ B cells detected by FACS analysis and the magnitude (number and size) of GC reactions or the presence of cells bearing non-IgM/IgD isotypes observed microscopically after immunohistochemical staining of tissue sections.

2: *Tissue fragment cultures to evaluate the immune status of the small intestine.*

Heretofore, humoral mucosal immune responses in animal models have been evaluated by determining antibody titres in secretions (milk, saliva, tears, tracheal lavage, intestinal washes, etc.) or, much less commonly, by enumerating antigen-binding, IgA plasma cells in sections or cell suspensions of intestinal LP. These are cumbersome and tedious procedures and the former suffers from problems of uncertain dilution, need for internal standards, and the possibility of enzymatic degradation of antibodies. We have developed a simple PP fragment culture and a complementary (small intestinal) lamina propria fragment culture which we believe accurately reflect the immune status of the small intestine at the time the tissue is sampled with respect to displaying a humoral mucosal immune response (*Logan* et al., 1991; *Kramer* and *Cebra*, 1992; *Shroff* and *Cebra*, 1993). At varying times following oral (or intraduodenal) exposure to antigens, animals are sacrificed, their PP are dissected from small intestine, fragments of these tissues are extensively washed in antibiotic containing solution and these are cultured at high O_2 for up to 10 days (*Logan* et al., 1991). Net increase in specific IgA antibodies from day 1 to day 7-10 culture fluids attest to specific mucosal responses and the continued viability of cultures. Total Ig and IgA output of these cultures provides an internal standard against which the specific responses may be normalised. Generally, LP cultures exhibit a 2-3 day lag over PP cultures in the time course of specific IgA antibody responses (*Kramer* and *Cebra*, 1992).

3: *A dispersed B cell microculture which scores and distinguishes antiRen-specific IRA memory cells and IRA-preplasmablasts based on their secretion of IRA antibody in single cell or clonal cultures.*

One of our major objectives has been to establish single B cell clonal microcultures that would enable assay of the functional potential of subsets of B cells to produce IgA. Heretofore, only the *ex vivo* splenic fragment culture has permitted the successful clonal outgrowth of IgA memory cells and this type of culture has not allowed definition of cellular interactions and CK requirements for the expression of IgA. In addition, no *in vitro* cultures heretofore available have supported the expression of IgA secretion by preplasmablasts from GCs.

Our original T/B microculture was based on clonal culturing of B cells responsive to thymus-independent antigens (*Schweitzer* and *Cebra*, 1988), as practised by the Nossal laboratory using antigen-specific B cells enriched by panning on haptenated gelatine (*Nossal* and *Pike*, 1978), except that we used cloned, antigen (conalbumin)-specific D10.G.4.1 T_H2 cells and haptenated antigen as stimuli (*Schrader* et al., 1990). Small numbers (10-20) of enriched, antigen-specific B cells give antibody secreting clones when placed in microcultures (10 μL) with non-limiting numbers of T_H2 cells (1500-3000) and their specific antigen conjugated to the hapten corresponding to the B cell specificity. These responses are clonal, Ia haplotype restricted, and exhibit requirements for hapten-carrier linkage if the T_H cells are 'rested' prior to use by brief (48 hr.) culture in the absence of antigen, APC, and LKs. The resting period results in a marked decline in cytoplasmic mRNA for IL-4 and IL-5 as detected by *in situ* hybridisation. An antigen-independent, haplotype-restricted version of these clonal microcultures has been developed using inputs of 0.5-2 purified F_1, k x b haplotype, B cells and the alloreactivity of

D10 cells vs. I-A^b. In either type of microculture, primary B cells can be stimulated to proliferate and generate clones that display isotype switching. However, IgA expression is rare among the clonal antibody products (6 ± 2% of antibody-secreting clones express IgA (*Schrader* et al., 1990). Further, B cells shown to include many IgA-memory cells, such as PP B cells enriched on phosphocholine (PC)-gelatine and tested in splenic fragment cultures, failed to generate clones secreting solely IgA in microculture (*Schrader* et al., 1990; *George* and *Cebra*, 1991). Even addition of exogenous IL-5 and IL-6 failed to markedly enhance IgA expression in clonal microculture and addition of TGFβ markedly reduced the cloning efficiency of B cells without increasing IgA expression (*Cebra* et al., l991a).

In an attempt to make T/B microcultures more supportive of IgA expression, we added either peritoneal macrophage, NIH/3T3, BALB/3T3, or dendritic cells (DC) as 'filler' or 'feeder' cells (*Schrader* et al., 1990; *Schrader* and *Cebra*, 1993). Although all types of added cells increased the frequency of responding B cells, only DC prepared from either spleen by the Steinman procedure or from PP by the Spalding method markedly potentiated IgA expression in both antigen-dependent and allo-stimulated clonal B cell microcultures. We found that as few as 400 DC per culture resulted in a marked increase in the proportion of antigen-dependent clones making anti-PC or anti-GlcNAc antibodies of the IgA isotype (to 30-50%). The IgA antibody-secreting clones included a sizeable fraction that secreted IgA antibodies exclusively (*Schrader* et al., 1990). A similar result was found for allostimulated B cells when DC were added (*Schrader* et al., 1990; *George* and *Cebra*, 1991). Neither the source - spleen or PP - of either the B cells or the DC affected the clonal expression of IgA when DC were added to the culture. If $sIgD^+$ B cells were prepared from either spleen or PP by FACS, then addition of DC to clonal microcultures resulted in a large fraction of clones that expressed IgA as one of several isotypes of antibody or Ig. Thus, addition of DC to these cultures revealed intraclonal isotype switching to IgA (*Schrader* et al., 1990).

Using allostimulated PP B cells we were also able to demonstrate that addition of DC to about one B cell and T_H cells allowed the outgrowth of IgA memory cells (*George* and *Cebra*, 1991). These IgA memory cells partitioned into the non-GC subset of PP cells prepared by FACS with a surface phenotype of low levels of peanut agglutinin binding (PNA^{low}) and high levels of sIg (kappa chain) ($s\kappa^{high}$). They were also markedly enriched by selecting the $sIgA^+$ cell fraction by FACS. When plated into alloreactive T_H-dependent microcultures containing DC these IgA memory cells gave clones that exclusively secreted IgA (*George* and *Cebra*, 1991).

Other features of the DC effect on T-dependent microcultures were that: (1) TH cells were required for generating antibody-producing clones, but their division during the culture period was not necessary as 2,000 R irradiation did not affect their efficacy; (2) the input of T_H cells could be reduced from about 3,000 without DC to about 375 with DC and comparable frequencies of responding clones were observed; and (3) the presence of DC promoted more vigorous cultures which survived longer and produced more total antibody than in their absence.

Although germinal centres developing in lymphoid tissues local to sites of antigen stimulation have been implicated as sites of generation of pre-plasmacells and memory B cells (*Cebra* et al., 1991b), direct demonstration of this role

has been frustrated by our inability to assay the functional potential of GC B cells. Necessary frequency analyses for specificity and isotype potential of GC B cells *in vitro* have heretofore been unsuccessful - GC cells usually die rapidly in culture - and assessment of their functional potential by adoptive transfer has been confounded by the lack of homing receptors on most GC B cells and the impossibility of defining the exact surface phenotype of progenitor cells responsible for any Ig expression in the host (*Cebra* et al., 1991b). Thus, when we developed a microculturing technique supportive of IgA expression we applied it to GC B cells from PPs, purified by FACS to enrich for PNA^{high} $s\kappa^{low}$ cells (*George* and *Cebra*, 1991). These cultures included DC and allospecific D10 cells reactive with $I\text{-}A^{b}$ molecules on the 0.5-2 PP GC cells ($I\text{-}A^{b/k}$) added. Under these conditions we found relatively low frequencies of responding cells and these gave clones that were not particularly distinctive of the PP site: Secretion of IgM, IgG1, and, only occasionally some IgE and IgA. Since about half to two-thirds of PP GC B cells from conventionally reared mice bore sIgA, we supposed that these were not responding with Ig secretion in our microcultures. However, we did find that our microcultures allowed extensive proliferation of GC B cells (*George* and *Cebra*, 1991). Because proliferation and differentiation have often been observed to be seemingly antagonistic processes, we assessed the effect of blocking division on Ig secretion by GC B cells from PPs (*George* and *Cebra*, 1991). If division of GC B cells is blocked by either x-irradiation (1600 R) or aphidicholin, a specific inhibitor of DNA polymerases, a high proportion of the positive cultures (60-70%) exclusively express IgA. About 30-40% of the division-blocked GC B cells responded with Ig secretion and both DC and T_H2 cells are required for such expression. Incorporation of ^{3}H-thymidine by B cells in such cultures is, of course, blocked and Ig-positive cultures expressing multiple Ig isotypes become rare. Experiments demonstrating GC B/T_H2 haplotype restriction and assay of secreted IgM by the F_1 input B cells using anti-allotypes strongly argue against B cell contaminants of the DC being the source of IgA in these cultures (*George* and *Cebra*, 1991). Finally, purification of $sIgA^{+}$ cells from PPs, distributed about 1:1 between GC and non-GC populations, enriches for both the uncommon memory IgA B cells (13-14%) that give clones exclusively secreting IgA in the standard microculture [see above] and also, especially for those that secrete IgA only when their proliferation is blocked. We hypothesise that our microculture assay of GC B cells from PPs, when their division is blocked, reveals a subset of cells committed to secretion. Such cultures demonstrate that, although many GC B cells die *in situ* by apoptosis, death need not be their immediate fate if their division is blocked and they are provided with necessary supportive cells and signals. These cultures also emphasise the extraordinary preference for the expression of IgM and IgA isotypes by GC B cells in PP and support the role of PP GC in the process of selective isotype switching to IgA expression.

4: *Assay for coating of gut bacteria* in vivo *with host IRA antibodies*.

Recently, we have begun to employ a rather simple but elegant assay for the occurrence, waxing, waning, continuation, or reappearance of gut mucosal IgA responses against bacterial colonisers. This assay was developed by Drs. D. and L. van der Waaij, Groningen, for human patients (personal communication). Faecal samples are periodically

collected, the bacterial suspension is washed, stained for IgA antibodies, and analysed by FACS. In our case, where colonisers consist of a single or few, known bacterial species the assay is more readily interpretable and permits non-destructive, constant monitoring of the gut mucosal IgA response.

RESULTS

The gut humoral, mucosal immune response to Gram-negative bacterial colonisers

We sought to determine and compare the effectiveness of various enteric bacteria at stimulating a mucosal IgA response and of cholera toxin (CT) at potentiating some of these responses. *Potter* (1971) had found that *M. morganii*, an occasional Gram-negative commensal of mice, reacted with certain anti-phosphocholine (PC) monoclonal antibodies and we had used this organism to colonise germfree mice and stimulate the appearance of specific anti-PC IgA memory cells in their PP (*Cebra* et al., 1980). Two, putative samples of *M. morganii* were used to successfully colonise GF mice within a few days after oral administration. PP fragment culture analyses of formerly GF mice mono-associated with one of these samples - now reclassified as *Proteus rettgeri* - failed to detect any specific anti-PC response up to 10 weeks after colonisation (*Logan* et al., 1991; *Shroff* and *Cebra*, 1993). FACS analysis failed to show any indication of GC reactions. Cholera toxin, a potent mucosal antigen in its own right, has been found to potentiate mucosal IgA responses to unrelated antigens when orally co-administered (*Elson* and *Ealding*, 1984). When the unresponsive, mono-associated mice were given 50 μg of CT orally, 6 weeks after colonisation, they made a prompt mucosal IgA response to CT as well as to the PC-determinant of their enteric bacteria. This response, detected in PP fragment cultures, was accompanied by obvious GC reactions in the PP. GF mice colonised only with the other sample of *M. morganii* - the authentic culture - made a prompt mucosal IgA anti-PC response at 10-14 days, as shown by PP fragment cultures (*Shroff* and *Cebra*, 1993) and FACS analyses of their PP cells. Figure 1 shows the waxing and waning of PNA^{high} GC cells over a 28-day period. Of note is that the colonisation at high bacterial density persists in these mice. So, having obtained a specific anti-PC IgA antibody response in PP fragment cultures of *M. morganii* immunised mice, we attempted to analyse the events leading to IgA commitment in GCs and the relationship between generation of IgA memory and secretory plasma cells. Roughly 40% of $sIgA^+$ cells in PP have elevated mRNA levels for alpha chain of IgA and most of these are PNA^{high} (*Weinstein* et al., 1991). It is likely that the survivors amongst these become secretory IgA plasma cells. $sIgA^+$ B cells from PP should contain both GC B cells that have switched to IgA expression (pre-plasmablasts) and non-GC, IgA memory cells. Using flow cytometry we enriched for PNA^{high} cells, and separately, for $sIgA^+$ cells from formerly GF mice mono-associated with *M. morganii* 15 days previously. The enriched cells were analysed in clonal B cell microcultures. Our findings were (*Shroff* and *Cebra*, 1993) that:

1. PC-specific B cells are present in both the PNA^{high} and $sIgA^+$ subsets at rather high frequencies at the time of maximal GC reactions in PP (~ 1%);
2. IgA memory cells produce clones expressing only IgA in T/B/DC cultures of some $sIgA^+$ B cells; and

3. Such IgA memory cells are rare in cultures of PNA^{high} enriched cells but both these and some sIgA^{+} cells give secreted IgA antibody when put into T/B/DC cultures in the presence of aphidicholin to block their division, prevent apoptosis, and permit IgA secretion.

We harvested *M. morganii* from the intestine of formerly GF mice at the time of maximal GC reactions (day 15). These were stained for host IgA coating their surface. Faecal *M. morganii* stained for host IgA, cultured *M. morganii* coated in vitro with MPC603, a monoclonal antibody against the PC-determinant of *M. morganii* also stained for IgA, and a control sample of cultured bacteria only stained with fluorochrome-labelled anti-murine IgA was negative. The FACS analysis indicates that a significant proportion of faecal bacteria have become endogenously coated with murine IgA.

We expect that a detailed comparison of murine host responses to gut colonisation with these two very closely related enteric bacteria at the single cell level and exploitation of this first example of the use of CT to overcome non-responsiveness to a chronically present enteric antigen, may provide some insight into a long-standing mystery: why the host does not appear to continuously respond to its commensal bacteria. Further, more comprehensive, functional assays, using clonal B cell microculture, should be informative of the quantitative and temporal development of antigen-specific IgA memory cells and pre-plasmablasts in relationship to the GC reaction in PP.

Are neonatal PP competent to generate a preferential IgA response?

Reoviruses given intraduodenally or orally cause sub-clinical enteric infections in immunocompetent adult mice but are potent stimulators of a mucosal IgA antibody response (*London* et al., 1987). Type 1 reovirus has a tissue tropism for M-cells overlying PP and can generate a rapid response in conventionally reared or GF mice as evidenced by PP fragment cultures (*Weinstein* and *Cebra*, 1991). These cultures generate specific antibody when established 3-6 days after *in vivo* infection and show peak responses if initiated 6-14 days post infection. We have previously found that neonatal mice show a delay in the development of IgA memory B cells vs. bacterial determinants associated with their normal gut flora; the frequencies of these specific IgA memory cells do not reach adult levels until 10-12 weeks of life (*Cebra* et al., 1986). In order to determine whether this delay was due to a delay in the development of fully functional PPs, able to confer preferential switching to IgA expression on locally stimulated B cells, we challenged 10 day old neonatal mice orally with reovirus 1 and compared their responses to 12 week old mice using PP and LP fragment cultures (*Kramer* and *Cebra*, 1992). Ten-day-old mice are the youngest that can contain reovirus infections to the gut and forestall often fatal sequelae such as hepatitis, meningo-encephalitis, biliary atresia or severe diarrhoea. A comparison of the fragment cultures clearly showed the development of a mucosal IgA response, peaking at about 6 days in PP followed by a progressive rise in LP, and that the time course was the same in 10 day or 10-12 week old mice (*Kramer* and *Cebra*, 1992). We next sought to examine the influence of the maternal immune system on the development of humoral mucosal immunity by the pups. Using reciprocal crosses of congenic BALB/c and CB.17 scid mice we have generated immunocompetent F1 pups that are born to and reared by either immunocompetent BALB/c or immuno-

incompetent CB.17 scid dams. Upon infecting both groups orally with active reovirus 1 we found no differences in the magnitude or kinetics of the developing, reovirus specific IgA antibody responses in PP or LP cultures (*Kramer* and *Cebra*, 1992). PP cultures from both groups produced virus specific IgM when initiated 3 days post infection (p.i.) and began to produce specific IgA if cultured at day 6 p.i. Control, non-challenged littermates of both groups were consistently negative for reovirus-specific antibodies. However, the non-challenged F_1 pups of BALB/c immunocompetent dams did not exhibit detectable 'total' or 'non-specific' IgA Ig in either PP or LP cultures until days 19-22 of life, while non-challenged pups born to CB.17 scid dams made detectable total IgA as early as day 13 of life (*Kramer* and *Cebra*, 1992). In close correlation with this finding, pups born of scid mothers had abundant LP and MLN IgA plasma cells by days 19-22 while pups born of immunocompetent mothers had very few (*Cebra* et al., 1993).

Of relevance to this present discussion was our observations that reovirus infected F_1 pups born of immunocompetent mothers also showed a premature increase in output of 'natural' IgA, similar to the spontaneous earlier appearance of 'natural' IgA exhibited by pups born of scid mothers. Presently, we are making similar reciprocal crosses to those described above except we are using GF parents. Mono-association of dams and pups with a particular bacterial coloniser should allow us to evaluate whether reovirus infection of neonates can overcome a specific suppression mediated by maternal antibodies or whether the virus infection potentiates a non-specific/polyclonal IgA response.

To further evaluate the role of maternally acquired passive immunity to reovirus in orally challenged neonates, dams were pre-immunised with different serotypes of reovirus by different routes (*Cuff* et al., 1990). Pups were challenged at 48 hr. after birth with Type 3 reovirus, which ordinarily causes death via meningo-encephalitis of all pups within 10 days ($3x10^6$ PFU/dose). Female mice immunised with homotypic virus via the oral route developed the most potent response. Infected neonates born and nursed by these females developed no signs of disease, and no virus was recoverable from their small intestines, livers, or brains following infection. Neonates born to females immunised with homotypic virus by the subcutaneous route manifested no evidence of meningo-encephalitis or virus dissemination, yet virus could be recovered from their intestines. Dams immunised with heterotypic virus by either the subcutaneous or the oral route also conferred protection vs. the fatal consequences of the disease. However, virus was recovered from both small intestines and livers of infected neonates. Based on results from foster-nursing experiments, it appears that factors obtained *both* during suckling and transplacental transfer contribute to protection. However, the most complete resistance to infection is via suckling on dams orally immunised with homotypic (serotype 3) virus.

A consequence of this protection is that pups from previously orally infected, immunocompetent dams do not exhibit an active mucosal IgA antibody response when they themselves are orally challenged. Foster nursing experiments indicate that this suppression of active, local mucosal immunity is not mediated by maternal antibodies delivered to the foetus before birth but rather by antibodies obtained during suckling.

Table 3: Transfer of BALB/c (Igh^a) PeC cells into non-irradiated, congenic CB.17 (Igh^b) adult or neonatal recipients

Age recipient[1]	#PeC transferred	Time to assay (days)	Donor B cells in PeC (%)	Tissue assayed[2]	IgA^a/ IgA (counts)[3]	IgA^a/ IgA (%)
Exp. 1						
adult	4x10^6	8	8	--	---	---
adult	4x10^6	13	5	LP	13/1016	1.2
adult	4x10^6	23	--	LP	2/1028	0.2
Exp. 2						
adult #1	5x10^6	1	28	--	---	–
adult #2	5x10^6	50	4	MLN	3/460	
adult #2	5x10^6	50	4	LP	14/181	7.6
adult #3	5x10^6	50	4	MLN	0/37	<3.0
adult #3	5x10^6	50	4	LP	55/3050	1.8
adult #4	5x10^6	85	--	MLN	0/38	<3.0
adult #4	5x10^6	85	--	LP	0/53	<2.0
adult #5	5x10^6	85	--	MLN	1/98	1.0
adult #5	5x10^6	85	--	LP	0/226	<0.5
Exp. 3						
neonate A	1x10^6	13	–	MLN	0/38	<3.0
neonate A	1x10^6	13	--	LP	0/524	<0.2
neonate B	1x10^6	13	--	MLN	1/61	1.6
neonate B	1x10^6	13	--	LP	0/1344	<0.1
neonate C[5]	1x10^6	13	--	MLN	0/0	–
neonate C[5]	1x10^6	13	--	LP	0/12	–
neonate D	2x10^6	16	53	MLN	0/23	<4.0
neonate D	2x10^6	16	53	LP	0/174	<0.6
neonate E	2x10^6	16	69	MLN	0/3	–
neonate E	2x10^6	16	69	LP	0/93	<1.0
neonate F[5]	2x10^6	16	26	MLN	0/0	–
neonate F[5]	2x10^6	16	26	LP	0/10	–
neonate G[5]	2x10^6	16	69	MLN	0/0	–
neonate G[5]	2x10^6	16	69	LP	0/23	<4.0

[1] Adult recipients 6-8 months of age; neonates 5 days old.
[2] MLN = mesenteric lymph nodes; LP = intestinal lamina propria.
[3] By fluorescence microscopy on cytospots with 10^5 cells/spot.
[4] Zynaxis dye not detectable but IgM^a B cells detectable in PeC.
[5] Assay of neonates done on groups of three, pooled; the neonates in these groups born of CB.17 (scid/scid) male x CB.17 (scid/scid) female. Groups A-C had 3 IgM^a/430 IgM (0.7%) and D-G had 8 IgM^a/193 IgM (1.9%) in spleens.

An attempt to evaluate the contribution of B1 B cells to the population of IRA plasma cells in the gut lamina propria

In an attempt to estimate the normal, physiologic contribution of B1 (PeC) B cells to the pool of IgA plasma cells in mesenteric lymph nodes (MLN) and LP of immunocompetent mice, we transferred inocula of BALB/c (Igh^a) peritoneal cavity (PeC) cells into congenic CB.17 (Igh^b) recipients. Both Ig-allotype markers and Zynexis vital dye labelling were used to determine the success and extent of engraftment.

Table 3 shows that adult recipients of inocula sufficient to account for an appreciable proportion of PeC B cells in

the host displayed, with one exception, only a few percent of MLN or LP IgA plasma cells of donor origin at 13, 23, 50, and 85 days after transfer. Donor PeC B cells could be detected in the PeC of recipients up to 50 days after transfer.

Newborn mice have few IgA or IgM plasma cells in MLN or LP for the first three weeks of life if they are born of immunocompetent mothers (F_1 from CB.20 [scid/scid] male x CB.17 [+/+] female. Neonates born of immunocompromised mothers (F_1 from CB.20 [+/+] male x CB.17 [scid/scid] female) exhibit appreciable numbers of IgA plasma cells in MLN and LP by two weeks of life (*Kramer* and *Cebra*, 1992; *Cebra* et al., 1993). We transferred 1-$2x10^6$ BALB/c PeC (Igh^a) cells, into five-day-old F_1 neonates derived from these reciprocal crosses. Table 3 shows that, although engraftment was successful, the transferred PeC B cells made little if any contribution to the developing population of MLN or LP IgA plasma cells after 13 or 16 days (by 18-21 days of life).

Congenic transfers of PeC B cells into immunocompetent adult or neonatal (5 day old) recipients suggest a quantitatively minor role for B1 B cells in maintaining the steady state level of IgA plasma cells in the MLN and intestinal lamina propria of immunocompetent mice. A caveat is whether the congenic host treats the Igh allotype-different inocula as 'self' or not.

DISCUSSION

For decades we have all pondered the enigma of non-or hypo-responsiveness of the host to copious, ever-present commensal bacteria (*Berg* and *Savage*, 1975; *Dubos* et al., 1965; *Wold* et al., 1989) and food antigens (*Wold* et al., 1989; *Thomas* and *Parrot*, 1974; *Nedrud* and *Sigmund*, 1991). Of course, this hypo-responsiveness may only be apparent, since most studies fail to comprehensively assess all elements of both the mucosal and systemic immune response. Alternatively, if real, the anergy may be constitutive - i.e., dependent on actual delivery of antigen across the gut mucosa - or it may be specifically inducible - i.e., dependent on the occurrence of a prior humoral and/or cellular immune response. We believe that our analyses of GF, AF, and CNV neonatal mice indicate a major role for the intestinal flora in the development and maintenance of the physiologically normal steady state of the elements of the mucosal immune system in the Peyer's patches, intestinal lamina propria, and the intraepithelial leukocyte compartment (IEL). However, our observations suggest a subtle distinction between the continuously ongoing activity of the humoral and cellular mucosal responses in the gut and what is displayed by spleen or peripheral lymph nodes upon chronic stimulation with the same set of antigenic determinants due to a persistent infection or repeated, deliberate parenteral introduction of antigens. We have observed a waxing and waning of GC reactions in PP of formerly GF mice similar to what one detects in draining lymph nodes of parenterally injected mice (*Kreal* et al., 1990; *Coico* et al., 1983). Such transient GC reactions are seen after oral reovirus infection, upon colonisation of GF mice with enteric bacteria, or after oral administration of CT (*Shroff* and *Cebra*, 1993), provided that the animals were maintained in otherwise protected isolators. Although the reovirus infection and CT antigens appear to be completely resolved or cleared, the coloni-

sation by enteric bacteria persists. Furthermore, re-exposure of the gut mucosa to the previously resolved antigens - such as reovirus - results in successively less robust GC reactions and humoral, mucosal immune responses (*Weinstein* and *Cebra*, 1991). These observations suggest that the humoral mucosal immune response works! Antigens are excluded, primary infection is prevented and the local mucosal immune system is shielded from intense restimulation. This outcome is very different from observations after secondary or tertiary stimulation of draining lymph nodes by parenteral introduction of antigens (*Kreal* et al., 1990; *Coico* et al., 1983).

The effectiveness of IgA antibodies in the gut lumen at shielding the local mucosal immune system from stimulation is supported by our analyses of neonates born of immune, immunocompetent dams versus pups born of non-immune and/or immuno-incompetent mothers. The former pups can be passively protected by suckling but their own active mucosal immune responses are forestalled by maternal antibodies. So, if pre-existing IgA antibodies in the gut lumen are so effective at forestalling local immune responses, why does the gut appear to be in a chronic state of inflammation and the PP, lamina propria, and IEL appear to be undergoing chronic stimulation? Several possibilities come to mind. One is that the chronically present GC reactions in PP of CNV mice may be maintained by an ever-changing array of gut luminal antigens, possibly supplied by new microbial colonists (or food antigens) or by antigenic variation of existing microbes. Our observations of the effect of CT on local responses to *P. rettergi*, after established colonisation, and of oral reovirus on the increase in 'natural' IgA output by neonatal gut suggest that occasional exposure to novel, effective gut mucosal antigens may activate or re-activate mucosal responses to 'bystander', currently ineffective antigens.

The normal, physiologic role of B1 B cells in contributing to the intestinal IgA plasma cell pool remains enigmatic. Although IgA or IgM plasma cells derived from anti-microbial specific (cross-reactive) B1 B cells presented an attractive possibility our present, imperfect efforts have not supported it. However, we have now developed an efficient system for generating IgA hybridomas in scid mice derived from $sIgM^{high}$, $sIgD^{low}$ B1 cells from peritoneal cavity or $sIgA^{+}$ B2 cells from PP. Recipient scid mice develop copious IgA plasmablasts in their mesenteric lymph nodes and these are effective fusion partners for generating hybridomas. Presumably, use of CNV scid or GF scid mouse hosts, and oral challenges with microbial colonisers or enteric viruses should allow us to assess the relative potentials of B1 and B2 B cells to contribute to mucosal, humoral immunity.

ACKNOWLEDGEMENTS

The work reported here has been supported by grants from the National Institutes of Health; AI-17997, AI-23970, and CA-09140.

LITERATURE

Berg, R.D. and Savage, D.C.: Immune responses of specific pathogen-free and gnotobiotic mice to antigens of indigenous and nonindigenous microorganisms. Infect.

Immun. 11, 320-329 (1975).
Bos, N.A., Bun, J.C.A.M., Meedendorp, B., Wubenna, A.S., Kroese, F.G.M., Ploplis, V.A., and Cebra, J.J.: B cell populations in antigen-free mice. In: Old Herborn University Seminar Monograph 6: The ontogeny of the immune system (Eds.: Heidt, P.J., Holmberg, D., Rusch, V., and van der Waaij, D.). Herborn Litterae, Herborn, 7-19 (2003)
Butcher, E.C., Rouse, R.V., Coffman, R.L., Nottenburg, C.N., Hardy, R.R., and Weissman, I.L.: Surface phenotype or Peyer's patch germinal center cells. J. Immunol. 129, 2698-2707 (1982).
Cebra, J.J., Gearhart, P.J., Halsey, J.F., Hurwitz, J.L., and Shahin, R.D.: Role of environmental antigens in the ontogeny of the secretory immune response. J. Reticuloendothel. Soc. 28, 61S-71S (1980).
Cebra, J.J., Fuhrman, J.A., Griffin, P., Rose, F.V., Schweitzer, P.A., and Zimmerman, D.: Changes in specific B cells and the dissemination of the primed state *in vivo* following antigenic stimulation by different mucosal routes. Ann. Allergy 53, 541-549 (1984).
Cebra, J.J., Cebra, E.R., and Shahin, R.D.: Perturbations in specific B cell subsets following deliberate contamination in germfree or natural colonization of neonatal mice by intestinal bacteria. In: Bacteria and the host (Eds.: Ryc, M. and Franek, J.). Avicenum, Prague, 303-307 (1986).
Cebra, J.J., George, A., and Kerlin, R.L.: A cautionary note concerning the possibility that transforming growth factor-beta is a switch factor that acts on primary B cells to initiate IgA expression. Immunol. Res. 10, 404-406, (199la).
Cebra, J.J., Schrader, C.E., Shroff, K.E., and Weinstein, P.D.: Are Peyer's patch germinal center reactions different from those occurring in other lymphoid tissue? Res. Immunol. 142, 222-226 (199lb).
Cebra, J.J., Bos, N.A., Cebra, E.R., DiLosa, R.M., Kramer, D.R., and Kroese, F.G.M.: Cellular and molecular biologic approaches for analyzing the *in vivo* development and maintenance of gut mucosal IgA responses. In Recent Advances in Mucosal Immunity: Proc. 7th Int. Congr. Mucosal. Immunol. (Eds.: McGhee, J.R., Mestecky, J., Tlaskalova, H., and Sterzl, J.). Plenum Publishing Corp., New York (1993).
Coico, R.F., Bhogal, B.S., and Thorbecke, G.J.: Relationship of germinal centers in lymphoid tissue to immunologic memory. VI. Transfer of B cell memory with lymph node cells fractionated according to their receptors for peanut agglutinin. J. Immunol. 131, 2254-2257, (1983).
Craig, S.W. and Cebra, J.J.: Peyer's patches: An enriched source of precursors for IgA-producing immunocytes in the rabbit. J. Exp. Med. 134, 188-200 (1971).
Cuff, C.F., Lavi, E., Cebra, C.K., Cebra, J.J., and Rubin, D.H.: Passive immunity to fatal reovirus serotype 3-induced meningoencephalitis mediated by both secretory and transplacental factors in neonatal mice. J. Virol. 64, 1256-1263 (1990).
Dubos, R., Schaedler, R.W., Costello, R., and Hoet, P.: Indigenous, normal, and autochthonous flora of the gastrointestinal tract. J. Exp. Med. 122, 67-75 (1965).
Elson, C.O. and Ealding, W.: Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. J. Immunol. 132, 2736-2741 (1984).
Forster, I. and Rajewsky, K.: Expansion and functional activity of Ly-1$^+$ B cells upon transfer of peritoneal cells into allotype-congenic, newborn mice. Eur. J. Immunol. 17, 521-528 (1987).
Fuhrman, J.A. and Cebra, J.J.: Special features of the priming process for a secretory IgA response: B cell priming with cholera toxin. J. Exp. Med. 153, 534-544 (1981).
Gearhart, P.J. and Cebra, J.J.: Differentiated B lymphocytes: Potential to express particular antibody variable and constant regions depends on site of lymphoid tissue and antigen load. J. Exp. Med. 149, 216-227 (1979).
George, A. and Cebra, J.J.: Responses of single germinal-center B cells in T cell-dependent microculture. Proc. Natl. Acad. Sci. USA 88, 11-15 (1991).
Hayakawa, K. and Hardy, R.R.: Normal, autoimmune, and malignant CD5$^+$ B cells: The Ly-1 B lineage. Ann. Rev. Immunol. 6, 197-218 (1988).
Kreal, G., Weissman, I.L., and Butcher, E.C.: Germinal centre B cells: Antigen specificity and changes in heavy chain class expression. Nature 298, 377-379 (1982).
Kramer, D.R. and Cebra, J.J.: Early IgA pro-

duction in suckling mice. In Abstracts: 8th Int. Congr. Immunol. Springer, Budapest, p. 46, Abstr. # 11-14, 1992.

Kroese, F.G.M., Butcher, E.C., Stall, A.M., Adams, S., and Herzenberg, L.A.: Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the peritoneal cavity. Int. Immunol. 1, 75-84 (1989).

Lalor, P.A. and Morahan, G.: The peritoneal Ly-1 (CD5) B cell repertoire is unique among murine B cell repertoires. Eur. J. Immunol. 20, 485-492 (1990).

Lebman, D.A., Griffin, P.M., and Cebra, J.J.: Relationships between expression of IgA by Peyer's patch cells and functional IgA memory cells. J. Exp. Med. 166, 1405-1418 (1987).

Linton, P.-J., Lo, D., Lai, L., Thorbecke, G.J., and Klinman, N.R.: Among naive precursor cell subpopulations only progenitors of memory B cells originate germinal centers. Eur. J. Immunol. 22, 1293-1297 (1992).

Logan, A.C., Chow, K.-P.N., George, A., Weinstein, P.D., and Cebra, J.J.: Use of Peyer's patch and lymph node fragment cultures to compare local immune responses to *Morganella morganii*. Infect. Immun. 59, 1024-1031 (1991).

London, S.D., Rubin, D.H., and Cebra, J.J.: Gut mucosal immunization with reovirus serotype 1/L stimulates specific cytotoxic T-cell precursors as well as IgA memory cells in Peyer's patches. J. Exp. Med. 165, 830-847 (1987).

McWilliams, M., Phillips-Quagliata, J.M., and Lamm, M.E.: Characteristics of mesenteric lymph node cells homing to gut-associated lymphoid tissue in syngeneic mice. J. Immunol. 115, 54-58 (1975).

Nedrud, J.G. and Sigmund, N.: Cholera toxin as a mucosal adjuvant: III. Antibody responses to nontarget dietary antigens are not increased. Reg. Immunol. 3, 217-222 (1991).

Nossal, G.J.V. and Pike, B.L.: Improved procedures for the fractionation and *in vitro* stimulation of hapten-specific B lymphocytes. J. Immunol. 120, 145-150 (1978).

Phillips-Quagliata, J.M.: Lymphocyte homing to mucosal effector sites. In: Mucosal Immunology, Vol. 1. (Eds.: Ogra, P.L., Lamm, M.E., Strober, W., McGhee, J.R., and Bienenstock, J.). Academic Press, San Diego (1992).

Pierce, N.F. and Cray, W.C., Jr.: Cellular dissemination of priming for a mucosal immune response to cholera toxin in rats. J. Immunol. 127, 2461-2464 (1981).

Pierce, N.F. and Gowans, J.L.: Cellular kinetics of the intestinal immune response to cholera toxoid in rats. J. Exp. Med. 142, 1550-1563 (1975).

Potter, M.: Antigen binding myeloma proteins in mice. Ann. NY Acad. Sci. 190, 306-321 (1971).

Riggs, J.E., Stowers, R.S., and Mosier, D.E.: The immunoglobulin allotype contributed by peritoneal cavity B cells dominates in SCID mice reconstituted with allotype-disparate mixtures of splenic and peritoneal cavity B cells. J. Exp. Med. 172, 475-485 (1990).

Schaedler, R.W., Dubos, R., and Costello, R.: Association of germfree mice with bacteria isolated from normal mice. J. Exp. Med. 122, 77-83 (1965).

Schrader, C.E., George, A., Kerlin, R.L., and Cebra, J.J.: Dendritic cells support production of IgA and other non-IgM isotypes in clonal microculture. Int. Immunol. 2, 563-570 (1990).

Schrader, C.E. and Cebra, J.J.: Dendritic cell dependent expression of IgA by clones in T/B microcultures. In: Dendritic cells in fundamental and clinical immunology. (Eds.: Hoefsmit, E.C.M., Kamperdijk, E.W.A., and Nieuwenhuis, P.). Plenum Press, New York, 59-64 (1993).

Schweitzer, P.A. and Cebra, J.J.: Quality of antibodies secreted by clones in microcultures from B cells enriched on haptenated gelatin: Isotypes and avidities. Molec. Immunol. 25, 231-241 (1988).

Shahin, R.D. and Cebra, J.J.: Rise in inulin-sensitive B cells during ontogeny can be prematurely stimulated by thymus-dependent and thymusindependent antigens. Infect. Immun. 32, 211-215 (1981).

Shroff, K.E. and Cebra, J.J.: Development of mucosal humoral immune responses in germ-free mice. In: Recent advances in mucosal immunity: Proc. 7th Int. Congr. on Mucosal Immunol. (Eds.: McGhee, J.R., Mestecky, J., Tlaskalova, H., and Sterzl, J.). Plenum Publishing Corp., New York (1993).

Taguchi, T., McGhee, J.R., Coffman, R.L., Beagley, K.W., Takatsu, K., and Kiyono, H.: Th1 and Th2 cells in mucosal associated sites. In: Advances in mucosal immunology (Eds.: MacDonald, T.T., Challacombe, S.J., Bland, P.W., Stokes, C.R., Heatley, R.V., and Mowat, A.McI.). Kluwer Academic Publishers, Dordrecht, 120-123 (1990).

Thomas, H. and Parrot, D.: The induction of tolerance to a soluble protein antigen by oral administration. Immunology 27, 631-639 (1974).

Weinstein, P.D., Schweitzer, P.A., Cebra-Thomas, J.A., and Cebra, J.J.: Molecular genetic features reflecting the preference for isotype switching to IgA expression by Peyer's patch germinal center B cells. Int. Immunol. 3, 1253-1263 (1991).

Weinstein, P.D. and Cebra, J.J.: The preference for switching to IgA expression by Peyer's patch germinal center B cells is likely due to the intrinsic influence of their microenvironment. J. Immunol. 147, 4126-4135 (1991).

Wold, A.E., Dahlgren, U.I.H., Hanson, L.A., Mattsby-Baltzer, I., and Midvetdt, T.: Difference between bacterial and food antigens in mucosal immunogenicity. Infect. Immun. 57, 2666-2673 (1989).