Old Herborn University Seminar Monograph 6:
The ontogeny of the immune system.
Editors: Peter J. Heidt, Dan Holmberg, Volker Rusch, and Dirk van der Waaij.
Herborn Litterae, Herborn-Dill, Germany: 7-19 (2003).

B CELL POPULATIONS IN ANTIGEN-FREE MICE

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SUMMARY

B cell populations in germfree mice fed a chemically defined ultrafiltered "antigen-free" diet (GF-CD) and conventional mice fed a diet of natural ingredients (CV-NI) are being investigated by immunophenotyping of the B cells using FACS analysis, and by immunohistological examination. The results show that small, follicular B cells and large, marginal zone B cells can be found in the spleen of GF-CD mice in numbers comparable to those in CV-NI mice. The numbers and sizes of mesenteric lymph nodes and Peyer's patches in GF-CD mice were smaller than in CV-NI mice and no germinal centres were detected. In the peritoneal cavity of GF-CD mice the number of B1 (Ly-1 B) cells is the same as in CV-NI mice and the B1 cells have the same level of IL5-receptor expression in both groups of mice. The most striking difference between GF-CD and CV-NI mice was the very severe reduction of IgA plasma cells in the lamina propria of GF-CD mice compared to that of CV-NI mice.

INTRODUCTION

The B cell repertoire can be subdivided into the potential, the available and the actual repertoire (*Coutinho* et al., 1984). The potential B cell repertoire is being formed by DNA rearrangements of germline genes encoding the heavy (V-D-J) and light (V-J) variable regions of immunoglobulins (*Early* et al, 1980). Only part of this potential Ig repertoire is represented among the available, immunocompetent B cells in the peripheral lymphoid organs. During an immune response some of the available B cells are being selected by antigens to either differentiate into Ig-secreting cells (actual repertoire) or to become memory B cells. By comparing the B cell repertoires in germfree mice fed a chemically defined synthetic diet (GF-CD) with conventional mice given a diet of natural ingredients (CV-NI), we can discriminate the endogenous and exogenous selective forces in the shaping of the B cell repertoire.

In the available repertoire, an influence of exogenous antigenic stimulation can be seen. In conventional mice the available splenic B cells have a seemingly "random" usage of V_H gene families in accordance with the size of the different V_H gene families (*Dildrop* et al., 1985; *Holmberg* et al., 1986; *Schulze* and *Kelsoe*, 1987). This "random" usage is probably antigen-se-

lected, because in adult conventional mice differences in V_H gene usage within a V_H gene family are observed between pre B cells in bone marrow and splenic B cells (*Gu* et al., 1991). In neonatal mice there is a biased usage of the V_H gene families that are most proximal to the constant Ig-region genes (*Holmberg*, 1987; *Bos* and *Meeuwsen*, 1989). A comparable biased usage was seen in adult GF-CD mice (*Bos* and *Meeuwsen*, 1989) and in GF mice (*Freitas* et al., 1991). The "normalisation" of V_H gene family usage in splenic B cells of adult conventional mice is thus apparently influenced by exogenous antigenic stimulation. The available repertoire in conventional neonatal mice and adult GF-CD mice is comprised of many B cells producing multireactive antibodies (*Bos* et al., 1989a). Such antibodies have also been reported to be produced by B1 cells (formerly called Ly1 B cells; *Kantor*, 1991) (*Hayakawa* et al., 1986).

GF-CD mice have a seemingly normal IgM production and a severely reduced production of IgG and IgA, as reflected by total numbers of Ig-secreting cells and serum Ig levels (*Bos* et al., 1988, 1989b). The frequency of antigen-specific IgG- and IgA-secreting cells greatly differs from that of the same isotypes in conventional mice, and is similar to that of the IgM-secreting cells (*Bos* et al., 1988, 1989b). Obviously, exogenous antigenic stimulation plays an important role in the development of the actual B cell repertoire.

Whether the described differences in the available and the actual B cell repertoire are reflected in differences in B cell populations between GF-CD and CV-NI mice is now being investigated by immunophenotyping of the B cells using FACS analysis, and by immunohistological examination.

Immunohistological staining of tissue sections and multiparameter flow-cytometric analysis of single cell suspensions of conventional murine tissues have demonstrated heterogeneity in the phenotype of B cells. For example, in immunohistological sections of the spleen of adult conventional mice at least two subpopulations of B cells are found: $IgM^{dull}IgD^{bright}$, which can be found in lymphoid follicles (follicular B cells or population I) and a much smaller $IgM^{bright}IgD^{dull}$ population, which predominantly can be found in the marginal zone (MZ) surrounding the follicles (MZ B cells or population III) (*Hardy* et al., 1984; *Kroese* et al, 1991). Upon antigenic stimulation so called germinal centres (GC) are formed in the follicles, containing large, rapidly dividing B cells. In addition to these B cell subsets a distinct lineage of B cells is formed by B1 cells (previously called Ly1 B cells). B1 cells differ developmentally from B cells which arise in the bone marrow (conventional B cells), as they are derived from foetal omentum and foetal liver (*Solvason* et al., 1991) and in the adult mice have a self-renewing capacity. They are predominantly found in the peritoneal cavity and exhibit unique phenotypic and functional properties (*Herzenberg* et al., 1986). Interestingly, MZ B cells and B1 cells in conventional mice share the same $IgM^{bright}IgD^{dull}$ phenotype and are both described as large cells, being in some state of "activation" (*Herzenberg* et al., 1986).

In this study we show that small, follicular B cells and large, MZ B cells can be found in the spleen of GF-CD mice in cell numbers comparable to those in CV-NI mice. The numbers of microscopically detectable MLN and PP in GF-CD mice were smaller than in CV-NI mice and no GC were detected.

In the peritoneal cavity of GF-CD mice and CV-NI mice both conventional B cells and B1 cells were found and the B1 cells have identical levels of IL5-re-

ceptor expression in both groups of mice. The most clear difference between GF-CD and CV-NI mice was the very severe reduction of IgA plasma cells in the lamina propria of GF-CD mice compared to that of CV-NI mice.

MATERIALS AND METHODS

Animals

BALB/cAnN mice were reared and maintained by American Biogenetic Sciences (Notre Dame, IN), either germ-free and fed a chemically defined ultrafiltered "antigen-free" diet L489-E14Se and LADEK 69E6 (GF-CD) as described in detail (*Pleasants* et al., 1986), or were maintained conventionally and fed natural ingredient diet L-485. CV-NI mice and GF-CD mice (in mini-isolators) were sent to the University of Groningen, The Netherlands, and kept under comparable conditions as in Notre Dame for four weeks before usage. Mice were fed their daily supply of fatty acids one hour before sacrifice.

Cell suspensions

Single cell suspensions from spleen were prepared in PBS, containing 10% NCS (PBS-NCS) by mincing tissue fragments over a stainless steel grid and filtering through a nylon mesh. Peritoneal cells were collected by rinsing the peritoneal cavity of adult mice with ca. 15 ml of PBS-NCS.

Antibodies

The following rat monoclonal antibodies were used: anti-IgM (331.12) (*Kincade* et al., 1981), anti-Ly1 (53-7.8) (*Ledbetter* et al., 1979), anti B220 (RA3-6B2) (*Coffman* and *Weissman*, 1981) and anti-IgA (71.14) (*Butcher* et al, 1982) and anti-IL5 receptor (*Rolink* et al., 1989) (a generous gift of Dr. A Rolink, Basel Institute for Immunology, Basel, Switzerland). For IgD staining a mouse anti-mouse anti-IgD^a was used (9.1) (*Stall* and *Loken*, 1984). Conjugation of antibodies to biotin and fluorescein has been described previously (*Hardy* et al., 1986).

Immunofluorescence staining and FACS analysis

Two-colour immunofluorescence staining of cells using FITC- and biotin-conjugated mAb was carried out in PBS-NCS as described in detail elsewhere (*Hardy* et al., 1984). Biotinylated antibodies were revealed by Streptavidine-Phycoerothrine (SA-PE; Southern Biotechnology Associates, Birmingham, AL) as second step reagent. Cells were analysed on a fluorescence activated cell sorter (FACStar; Becton Dickinson, Mountain View, CA). Non-lymphoid cells were gated out on the basis of forward and perpendicular site scatter pattern. For each analysis data from 10,000-20,000 cells were collected.

Immunohistology

Cryostat sections were prepared from spleen, MLN, PP and lamina propria. MLN and PP were only detectable in GF-CD mice by examination under a dissecting microscope, because of the white appearance of the lymph veins due to ingestion of fat one hour before. Staining of the cryostat sections with mAb using an indirect immunoperoxidase technique was performed as described elsewhere (*Kroese* et al., 1987). As second-stage antibody, peroxidase-conjugated polyclonal rabbit anti-rat Ig (Dakopatts, Copenhagen, Denmark) was used. For anti-IgD^a an Avidine-peroxidase conjugate (Southern Biotechnology Associates, Birmingham, AL) was used.

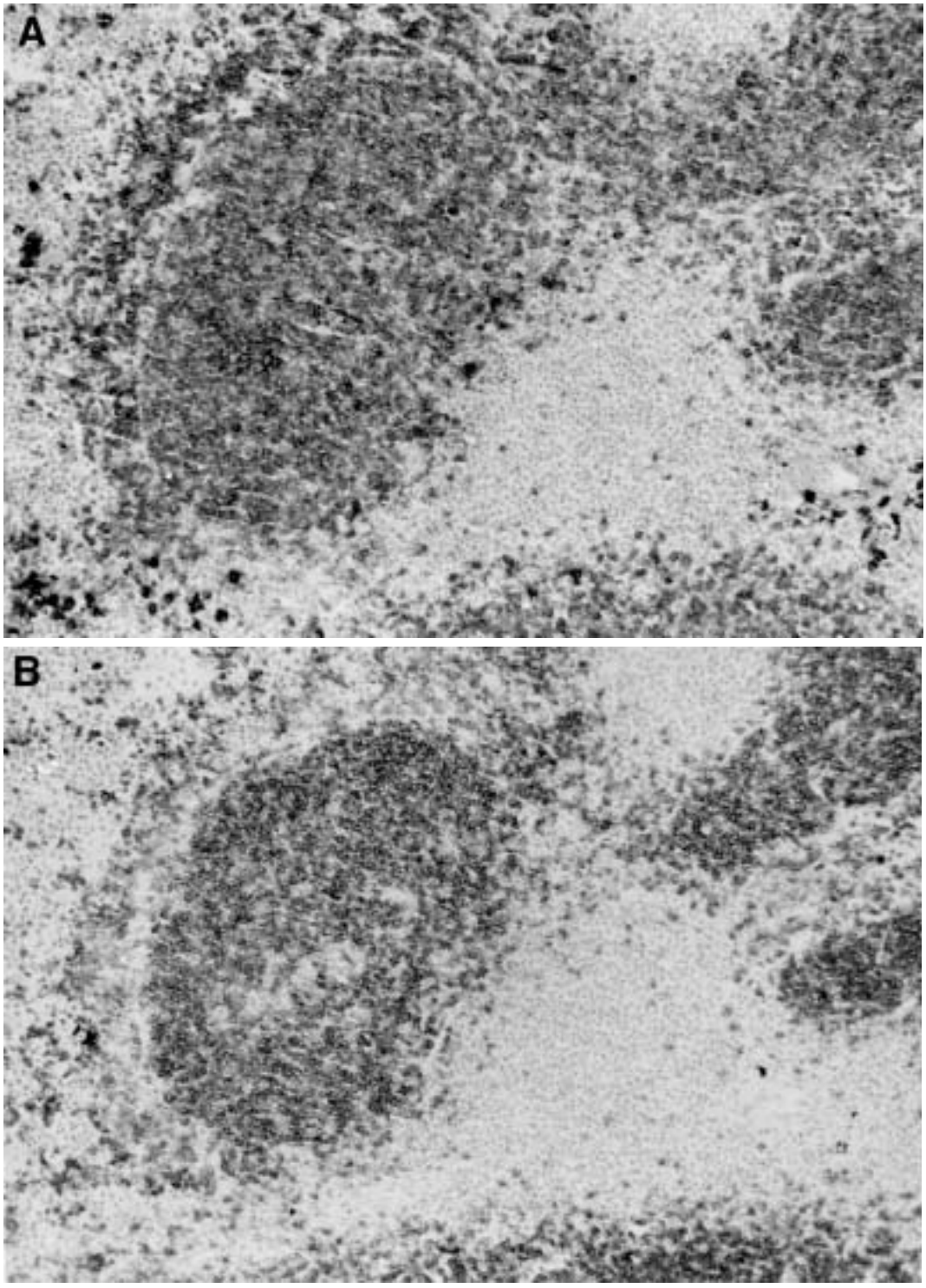

Figure 1: Serial cryostat sections of the spleen of a GF-CD mouse stained with (A) anti-IgM and (B) anti-IgD (x60).

Table 1: Flow cytometric analysis of B cell subpopulations in spleen and peritoneal cavity of GF-CD and CV-NI BALB/c mice.

Organ	phenotype	GF-CD	CV-NI
Spleen	IgD^{high}/IgM^{low} (follicular B cells)	47%[1]	52%
	IgD^{low}/IgM^{high} (MZ B cells)	8%	8%
Peritoneal cavity	IgD^{high}/IgM^{low} (conventional B cells)	14%	27%
	IgD^{low}/IgM^{high} (B1 cells)	68%	52%
	$Ly\text{-}1^{+}/IgM^{high}$ (B1a cells)	40%	29%
	$Ly\text{-}1^{-}/IgM^{+}$ (B1b+conventional B cells)	53%	62%
	$IgM^{+}/IL5R^{+}$	74%	71%

[1]Percentage represents the mean percentage of all lymphoid cells in the different organs of individually analysed 8-12 wk old GF-CD BALB/c mice (n=3) and CV-NI BALB/c mice (n=3).

RESULTS

B cell populations were examined by flow cytometry and/or immunohistology in spleen, MLN, PP, lamina propia and peritoneal cavity.

Spleen

In the spleen of conventional mice the majority of small resting, $IgM^{low}IgD^{high}$ B cells can be found in the follicles. Furthermore, large B cells, which are $IgM^{high}IgD^{low}$, can be seen in the marginal zone surrounding the follicles. Upon antigenic stimulation so called germinal centres (GC) are formed in the follicles, containing large, rapidly dividing B cells.

In the spleen of GF-CD mice essentially the same B cell subpopulations were observed by immunohistology, except for the total lack of GC. Figure 1 shows serial frozen sections of the spleen of GF-CD mice stained with anti-IgM (Figure 1a) and anti-IgD (Figure 1b). Follicles were present, surrounded by well-developed marginal zones. Furthermore, IgM plasma cells were found in the red pulp of the spleen of GF-CD and CV-NI mice (Figure 1a).

Also quantitatively the same numbers of $IgM^{low}IgD^{high}$ and $IgM^{high}IgD^{low}$ B cells were found by flow cytometry. In GF-CD and CV-NI mice there were 47% and 52% $IgM^{low}IgD^{high}$ B cells and 8% and 8% $IgM^{high}IgD^{low}$ B cells, respectively (Figure 2, Table 1).

Mesenteric lymph nodes

MLN are very hard to find in GF-CD mice, showing that their size is clearly dependent on exogenous antigenic stimulation. However, because of the rapid uptake of the fatty acids fed one hour before sacrifice, the lymph veins can easily be traced with use of a dissecting microscope. In this way we were able to find MLN in GF-CD mice. Immunohistological examination showed a normal architecture of these lymph nodes. A cryosection stained with anti-B220, a common B cell marker, showed normal follicular B cell areas (Figure 3). In MLN of CV-NI mice several GC were observed, while in GF-CD mice only one small, beginning GC was detected (data not shown).

Peyer's patches

Gut associated clusters of lymphoid follicles, known as Peyer's patches (PP) can be found along the intestinal tract of conventional mice. PP could only be detected in low numbers in GF-

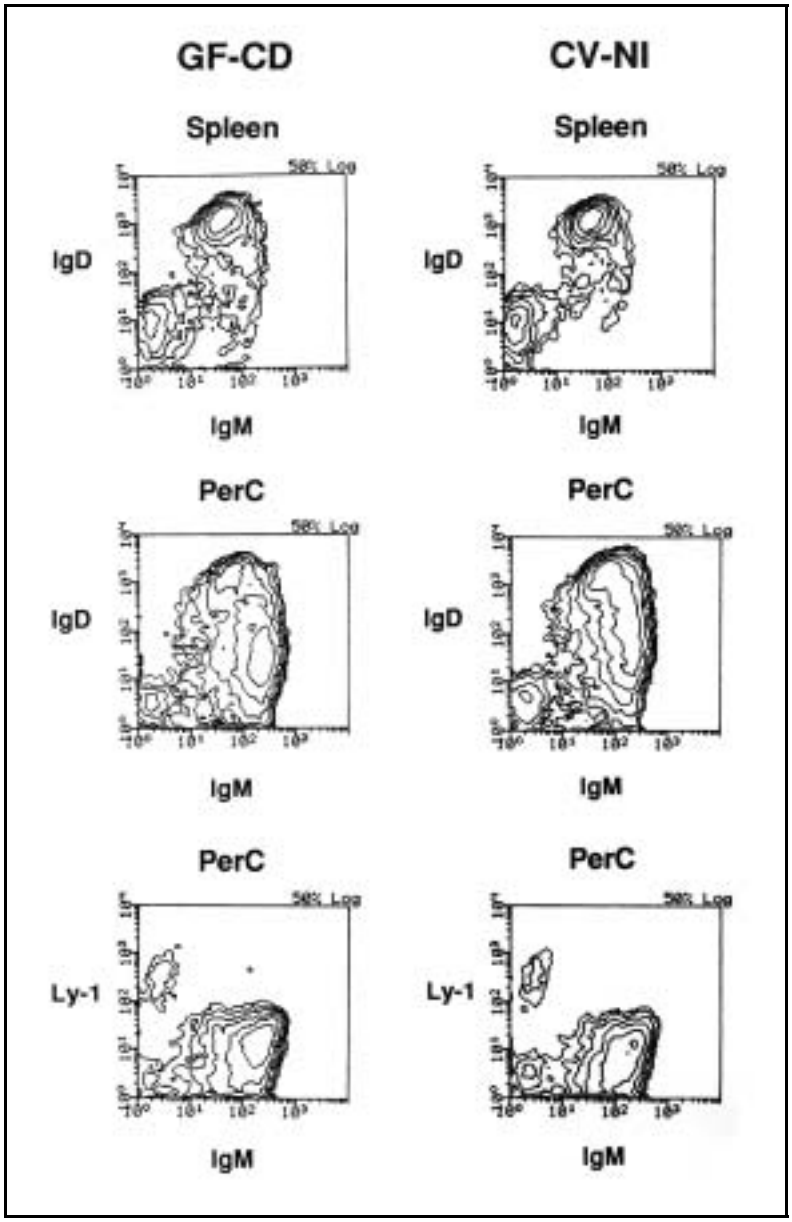

Figure 2: Two colour FACS analysis of splenic and peritoneal cavity (PerC) cell suspensions of 8-12 wk-old GF-CD and CV-NI mice, stained with combinations of anti-IgM/anti-IgD and anti-IgM/Ly-1. Figures show representative samples; calculations are shown in Table 1.

CD mice after careful examination of the gut with a dissecting microscope. The follicles of PP of CV-NI mice contained many GC with many sIgA-positive B cells (data not shown). By contrast, follicles of PP in GF-CD mice contained follicular B cells as shown by B220 staining (Figure 4) and IgM and IgD, but they did not contain GC and sIgA positive cells were absent.

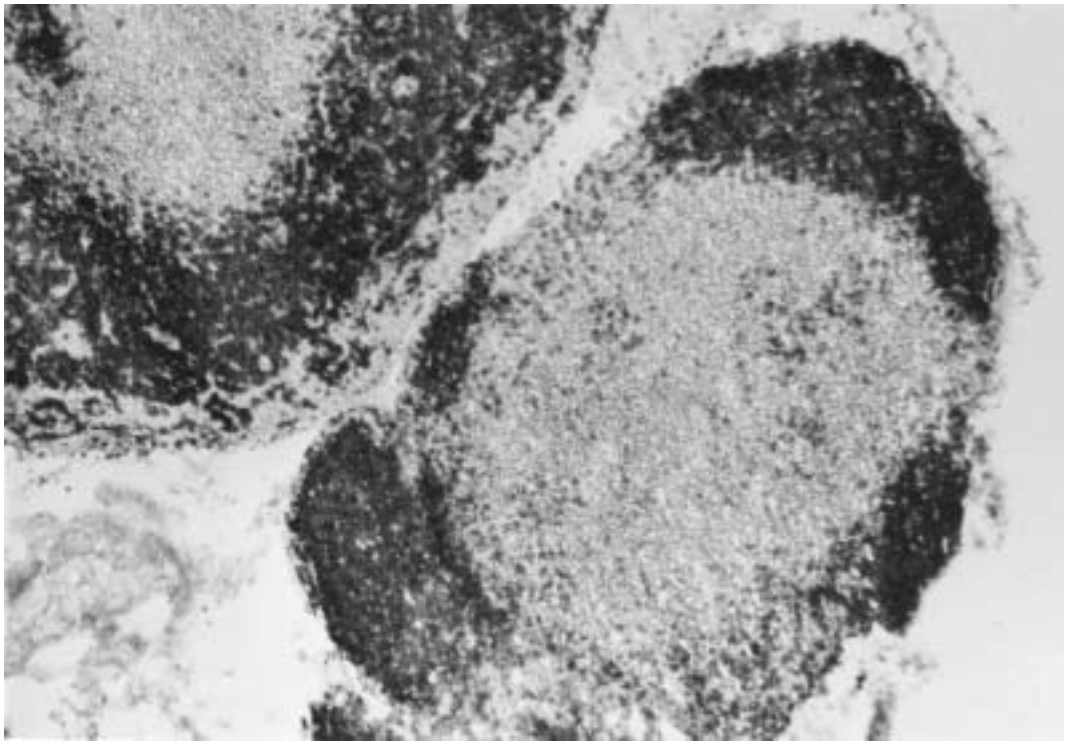

Figure 3: Cryostat section of a mesenteric lymph node of a GF-CD mouse, stained with anti-B220 (x60).

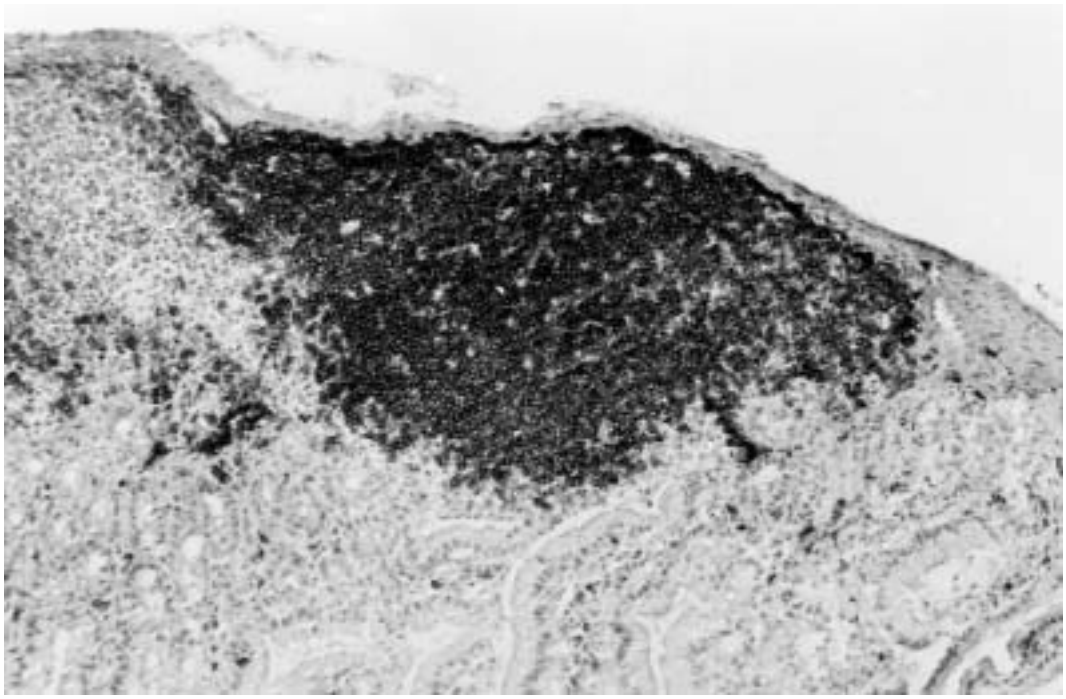

Figure 4: Cryostat section of a Peyer's patch of a GF-CD mouse, stained with anti-B220 (x60).

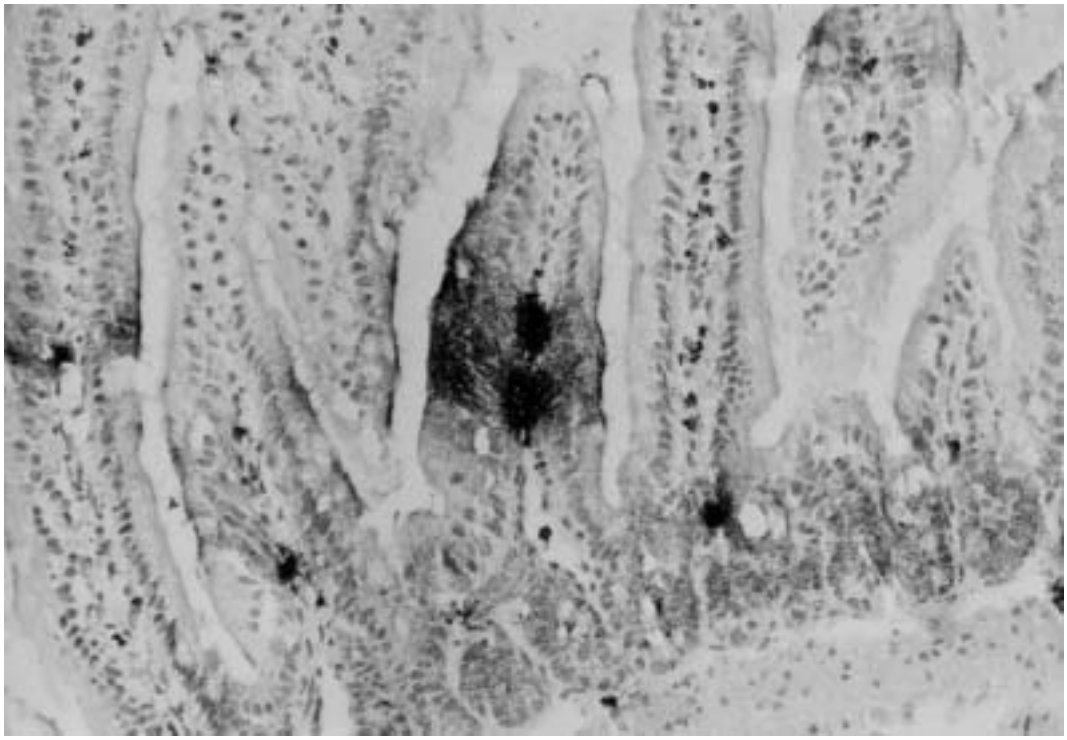

Figure 5: Cryostat section of the lamina propria of the gut of a GF-CD mouse, stained with anti-IgA (x100).

Lamina Propria

In the lamina propria of CV-NI mice very large numbers of IgA plasma cells were located (data not shown). Previous studies have shown that more than 80% of all Ig-secreting cells in conventional animals can be found in the lamina propria and these produce mainly IgA (*van der Heiden* et al., 1987). By contrast, the lamina propria of GF-CD mice contained extremely few IgA plasma cells (Figure 5) and no IgM plasma cells. In cryosections of the gut of GF-CD mice IgA can be detected surrounding the rare IgA plasma cells, suggesting the secretion of IgA into the intestinal tract (Figure 5).

Peritoneal cavity

In CV-NI mice high numbers of B cells are found in the peritoneal cavity. Most of the peritoneal B cells belong to a distinct lineage of B cells, called the B1 cells (formerly called Ly1 B cells), of which the majority express low amounts of CD5. The B1 cells in the peritoneal cavity have a characteristic $IgM^{high}IgD^{low}$ phenotype in contrast to conventional B cells that are $IgM^{low}IgD^{high}$. In GF-CD and CV-NI mice both conventional B cells and B1 cells were found. GF-CD and CV-NI mice contained 14% and 27% of $IgM^{low}IgD^{high}$ conventional B cells and 68% and 52% $IgM^{low}IgD^{high}$ B1 cells respectively (Figure 2 and Table 1). The Ly-1 (CD5) positive B1 cells (B1a cells) were 40% and 29% in GF-CD and CV-NI mice, respectively (Figure 2 and Table 1). IL5 receptor expression on peritoneal B cells was examined by double staining with anti-IgM and anti-IL5 receptor mAbs. Table 1 shows that both in GF-CD and in CV-NI mice a similar high percentage

of the B cells express the IL5 receptor (74% and 71%, respectively). There was a positive correlation between the level of IgM expression and IL5R expression, showing that IgM^{high} (B1 cells) had the highest expression of IL5 receptor (data not shown).

DISCUSSION

B cell populations in GF-CD mice

Frequencies of B cells and B cell subsets in various lymphoid organs such as spleen, lymph nodes and PP are independent of exogenous antigenic stimulation, since B cell follicles and the marginal zone surrounding the follicles in the spleen were the same in GF-CD and CV-NI mice. Also, the number of peritoneal B1 cells was comparable in both groups of mice. However, there were some differences observed between GF-CD and CV-NI mice.

Firstly, the number and size of gut-associated lymphoid tissues such as MLN and PP were severely reduced, but still detectable, in GF-CD mice compared to CV-NI mice. This finding is in contrast to that of others who failed to detect such organs in GF-CD mice (*Hooijkaas* et al., 1984; *Pereira* et al., 1986). This discrepancy is probably due to technical differences in the dissection procedure, since we only detected MLN in GF-CD mice if the animals had ingested fatty acids one hour earlier. Both these and our studies, however, clearly show that full development of gut- associated lymphoid tissue is dependent on exogenous antigenic stimulation.

Secondly, virtually no GC were found in the lymphoid organs of GF-CD mice, not even in the PP, where in normal animals many GC can be found. GC are thought to be the site where memory B cells are formed and affinity maturation and isotype switching of antigen-specific B cells takes place after immunisation (*Kroese* et al., 1990). Earlier studies with GF mice showed some GC formation in the MLN (*Pollard* et al., 1967). Probably this is due to the antigenic stimulation from antigens of the sterilised conventional food of such animals.

Thirdly, there were almost no sIgA-positive cells in the PP and only few IgA plasma cells were found in the lamina propria of GF-CD mice. This is in agreement with earlier findings in GF mice, which show also a clear reduced number of sIgA-positive cells in the PP (*Weinstein* and *Cebra*, 1991) and in the number of intestinal IgA-secreting cells (*van der Heiden* et al., 1989). The sIgA-positive cells in the PP are considered as precursors for both IgA plasma cells in the lamina propria and IgA memory cells (*Gearhart* and *Cebra*, 1979). Interestingly, there were still some IgA plasma cells found in the lamina propria of GF-CD mice. Recently, it has been found that not only PP cells can give rise to IgA plasma cells, but that peritoneal B1 cells are also a source for intestinal IgA-plasma cells (*Kroese* et al., 1989). Since GC are absent in the PP of GF-CD mice, possibly the IgA plasmacells in the gut of GF-CD mice belong to this lineage. Where and how the B1 cells get triggered to become IgA plasma cells is still unknown.

Available repertoire

In GF-CD mice and in GF mice a biased V_H gene family usage of the V_H gene family PC7183 that is most proximal to the constant Ig genes is observed (*Bos* and *Meeuwsen*, 1989; *Fre-*

itas et al., 1991). A comparable biased V_H gene family usage has been observed in neonatal B cells and in pre-B cells in the bone marrow of adult conventional mice (*Manlynn* et al, 1990). In adult conventional mice a more stochastic V_H gene family usage was seen according to the size of the V_H gene families (*Dildrop* et al., 1985; *Holmberg* et al., 1986). Also, within the large V_H gene family J558, differences were observed in V_H gene usage between bone marrow pre-B cells and splenic B cells, suggesting that the seemingly random usage in adult conventional mice is caused by antigenic selection (*Gu* et al., 1991). In GF mice, it was shown that injection of IgG derived from conventional mice, causes a normalisation of V_H gene family usage (*Freitas* et al., 1991). This normalisation could either be caused by direct polyclonal antigenic stimulation by the (foreign) injected IgG or IgA antibodies and/or by idiotypic-anti-idiotypic interactions of the injected antibodies with the B cells of the recipient. Until now the available repertoire has been studied in hybridomas after LPS stimulation (*Dildrop* et al., 1985; *Holmberg* et al., 1986; *Bos* and *Meeuwsen*, 1989), by analysis of the total RNA of spleen or bone marrow cells (*Manlynn* et al., 1990) or by *in situ* hybridisation (*Jeong* and *Teale*, 1988). The contribution of different B cell subpopulations in these assays is unknown. What are possible explanations for the differences between the available repertoire in adult GF-CD and CV-NI mice?

Firstly, memory B cells, which are considered to be immunocompetent B cells that can contribute to the available B cell repertoire. The lack of GC in GF-CD mice, suggests that the memory B cell repertoire in GF-CD mice is absent. This absence may explain (partly) the observed "normalisation" of the V_H gene family usage in conventional mice.

Secondly, a source for the differences in the available repertoire between adult GF-CD and CV-NI mice might be the MZ B cells. MZ B cells are relative large cells (*Herzenberg* et al., 1986; *Liu* et al., 1992). The contribution of MZ B cells to the analysed available repertoire is unknown. MZ B cells represent about 13% of the splenic B cells, but if they are preferentially stimulated by LPS or if these relatively large B cells contain more RNA than the small follicular B cells they may contribute significantly to the V_H gene family distribution as found among splenic B cells. This explanation is unlikely, because the number of MZ B cells seems independent of exogenous antigenic stimulation, as no differences were found by FACS analysis and immunohistology of MZ B cells in GF-CD and CV-NI mice.

Finally, the observed differences in the available repertoire between GF-CD and CV-NI mice might be due to a selection process of the follicular B cells. Where and how this selection takes place is still unknown.

Actual repertoire

Since memory formation and isotype switching may take place within the GC, the lack of GC in GF-CD mice is in agreement with our earlier findings that GF-CD mice have a severely reduced production of IgG and IgA, both at the level of Ig-secreting cells and at the level of serum Ig (*Bos* et al., 1988, 1989b). Furthermore, the specificity repertoire of the low numbers of IgG- and IgA-secreting cells greatly differs from that of the same isotypes in conventional mice, and is much like the IgM specificity repertoire (*Bos* et al., 1988, 1989b). Obviously, exogenous antigenic stimulation plays an important role in the development of the actual B cell repertoire. The emerging actual B cell repertoire after immunisation of GF-CD mice has not yet been investigated.

In this regard it is interesting that the production of monoclonal antibodies in GF-CD mice seems to be more efficient then in conventional mice (*Ploplis*, unpublished observations). GF-CD mice contain normal numbers of large, presumably activated, B cells in the spleen as judged by FACS analysis (*Pereira* et al., 1986). Furthermore, the numbers of IgM-secreting cells in spleen, BM and MLN is the same in GF-CD and CV-NI mice (*Pereira* et al., 1986; *Bos* et al., 1988). Also, the specificity repertoire of these IgM-producing cells was comparable between GF-CD and CV-NI mice (*Bos* et al., 1988). A possible source for these so called "natural" IgM antibodies could be the B1 cells. B1 cells are shown to be a separate lineage of B cells which can self renew largely independent of the bone marrow (*Herzenberg* et al., 1986). In transfer experiments with allotypic Ig markers it has been shown that this relatively small compartment of B cells (estimated to represent 1% of all B cells) can produce up to 50% of all serum Ig (*Kroese* et al., 1989). The B1 cells are relatively larger then conventional B cells and a large proportion of them express the IL5 receptor (*Wetzel*, 1989). They seem to be a selected B cell population, because they use a restricted set of Ig genes and limited specificities (*Hayakawa* et al., 1986). How they are selected is unknown. On the other hand, other studies showed no differences in the B1 cell specificity repertoire of GF and conventional mice, suggesting that the B1 cell specificity repertoire is established independent of exogenous antigenic stimulation (*Lalor* et al., 1989). In the GF-CD and CV-NI mice we showed comparable numbers of B1 cells and the same level of IL5 receptor expression. This is in agreement with others who found normal numbers of B1 cells in GF mice (*Forster* et al, 1991). If exogenous antigenic stimulation plays a role at all in the selection of the B1 cell specificity repertoire, it is not reflected in the number or phenotype of the B1 cells.

In conclusion, mice that are kept under conditions that will minimise exogenous antigenic stimulation, have a normal architecture of the B cell areas in the lymphoid organs and numbers of different B cell subpopulations that are similar to those of conventional mice. The available repertoire of adult GF-CD mice is different from that of conventional mice, suggesting a role for exogenous antigenic stimulation in the establishment of the available repertoire. The actual IgM specificity repertoire develops independent of exogenous antigenic stimulation. This is in contrast to the actual IgG and IgA repertoire, which is clearly influenced by exogenous antigenic stimulation. Reconventionalisation and immunisation of GF-CD mice can further clarify the role of exogenous antigenic stimulation in the shaping of the B cell repertoires.

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