THE INFLUENCE OF EXOGENOUS ANTIGENIC STIMULATION ON THE MURINE B CELL REPERTOIRE

DE INVLOED VAN EXOGENE ANTIGENE STIMULATIE
OP HET B CEL REPERTOIRE VAN DE MUIS

PROEFSCHRIFT

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CHAPTER 1

THE HUMORAL IMMUNE SYSTEM

1.1. General introduction

The immune system is characterized by its ability to recognize specifically foreign substances (antigens) and efficiently prevent infections. This ability is carried out by lymphocytes, which can be divided into two major categories, the B en T lymphocytes. The B lymphocyte has the ability to produce antibodies upon recognition of its specific antigen and thus accounts for the humoral immune response. T lymphocytes can recognize antigens only when they are presented by self-proteins of the major histocompatibility complex (MHC) on the membrane of an antigen-presenting cell (APC). T lymphocytes can functionally be divided into helper T cells which upon activation produce lymphokines which can activate and provide help for B and T cells, and cytotoxic T cells which are able to directly lyse their target cells, for instance virus-infected cells. Each lymphocyte has only one type of antigen-receptor which can recognize only a single or some structurally related antigenic determinants. For the recognition of the large spectrum of antigens a large number of different lymphocytes are needed. The total spectrum of antigen-receptors (on individual lymphocytes) is called the specificity repertoire of lymphocytes. The genetic mechanisms accounting for this large diversity have mostly been revealed during the last two decades. It was found that by rearrangement of different genetic elements and by other somatic mechanisms a large number of different receptors can be made. Within this large repertoire the potential of self-recognition is also included.

The immune system discriminates between self and non-self. T lymphocytes undergo selection in the thymus where only those lymphocytes which have the ability to recognize foreign antigens in combination with self MHC-molecules and which are not reactive with self antigens, are being selected. For B lymphocytes such a mechanism is not known. Autoreactive antibodies can be found in the serum of normal individuals and generally do not seem to be harmful, although in some autoimmune diseases self reactive antibodies play a central role.

The regulation of the B cell specificity repertoire is subject of this study. We investigated to what extent the B cell specificity repertoire is dependent on exogenous antigenic influences. This was studied in mice which are kept under germ-free conditions and given a chemically defined ultrafiltered (< 10.000 D) 'antigen-free' diet. These mice were compared to mice kept under conventional conditions. The study provides evidence for a subdivision of the B cells into two compartments. The first compartment of B cells produces multireactive, among which autoreactive, mostly IgM-

antibodies of low affinity and is established early in ontogeny. The second compartment of B cells is formed after exogenous antigenic stimulation and produces high affinity antibodies and constitutes the great majority of B cells at the adult age under normal circumstances. Under 'antigen-free' conditions only the first compartment develops and seems to be maintained throughout life. Autoreactive antibodies involved in autoimmune diseases most probably resort in the second compartment.

1.2. B cell differentiation

B lymphocytes are produced in fetal and adult hemopoietic tissues from pluripotent hemopoietic stem cells (Owen et al., 1974; Raff et al., 1976). In adult individuals the bone marrow is the major site of the generation of immunocompetent B lymphocytes (reviewed by Osmond, 1986). Mature B lymphocytes can be recognized based on the immunoglobulin (Ig) molecule and some other B-cell specific antigens on their surface membrane, like the surface molecule B220 (Kincade et al., 1981). The population of $B220^+$, surface Ig^- B cell progenitors can be subdivided on the basis of intracellulair markers. Firstly, it includes pre-B-cells expressing no Ig light chains, but μ-heavy chains detectable in their cytoplasm only (Raff et al., 1976; Levitt and Cooper, 1980). Secondly, it includes cells containing terminal deoxynucleotidyl transferase (TdT) which may represent part of the stages in bone marrow preceding μ chain expression (Park and Osmond, 1987). TdT is an intranuclear enzyme that supposedly contributes to somatic diversification of antigen receptors (Desiderio et al., 1984).

These bone marrow precursors are responsible for the production of minimally 5×10^7 newly formed B cells per day in the mouse (Osmond, 1986), which migrate to the peripheral organs like spleen and lymph nodes. In an adult mouse, the peripheral B cell pool of immunocompetent cells contains about 5×10^8 lymphocytes in a dynamic equilibrium. Most of these B cells are short-lived and have a high turnover rate ($\approx 3 \times 10^7$/day). A small proportion of the peripheral B cells ($\approx$ 10%) have a long-life expectancy (10-20 days or more) (Freitas et al., 1986). Some mature B cells differentiate into Ig-secreting cells (plasma cells), which produce the serum and secretory Ig. In an adult mouse approximately $1\text{-}2 \times 10^6$ Ig-secreting cells can be found in spleen, bone marrow and mesenteric lymph nodes (Benner et al., 1982). Furthermore, recent data have shown that about 16×10^6 IgA-secreting cells can be found in the small intestine, which probably mostly contribute to the IgA secreted into the intestinal lumen and to some extent to the serum IgA pool (Van der Heijden et al., 1987).

Every mature B lymphocyte expresses an Ig molecule on its cell membrane, which is unique in its variable (V) sequences. The variable part of the Ig-molecule can

bind specifically to antigen. According to the natural selection theory of antibody formation (Jerne, 1955), extended to the clonal selection theory of antibody production (Burnet, 1957, 1959), B cells are selected by antigen via their surface Ig receptors to proliferate and produce specific antibodies. The ability of an organism to make antibodies to antigens which it has never encountered before, depends on the repertoire of B cells with different antigen-receptors. This specificity repertoire of B cells can functionally be divided into different repertoires taking into account the various B cell differentiation stages (Coutinho et al., 1984):

1. The potential repertoire, determined by the number, structure and mechanisms of expression of the germline genes encoding antibodies plus the possible somatic variants derived from them. This repertoire is present in the germline of the stem cells which have not yet undergone Ig gene rearrangement.
2. The available repertoire, defined as the set of diverse antibody molecules that are expressed by immunocompetent but resting B cells.
3. The actual repertoire, represented by the set of Ig molecules that are actually secreted by Ig-secreting cells. This actual repertoire is also represented in the serum Ig pool.

The selection of the different repertoires during the dynamic process of B cell differentiation is caused by different factors. The different genetic mechanisms which eventually lead to the successful production of Ig-molecules will be explained in the next section. Complete Ig-molecules can be the target for regulation. Selection by antigen on the available repertoire of B cells obviously leads to the shaping of the actual B cell repertoire. Since every B cell has an unique, somatically formed, Ig molecule with unique antigenic determinants (idiotopes) it is envisible that other B cells can produce antibodies which recognize these structures. This had led to the hypothesis of the idiotypic network. The selection by antigens and the idiotypic network will be discussed in more detail in subsequent sections.

1.3. Generation of antibody diversity

1.3.1. Genetic basis of diversity

Ig molecules are made of two heavy (H) and two light (L) chains, each consisting of a constant (C_H and C_L) and a variable (V_H and V_L) part. The variable parts are responsible for antigen binding, while the constant part determines the Ig (sub)class or isotype (in the mouse: IgM, IgD, IgG1, IgG2a, IgG2b, IgG3, IgE and IgA) (Edelman, 1973; Jeske and Capra, 1984). Mouse Ig chains are encoded in three unlinked gene families: λ light-chain genes, κ light-chain genes and heavy chain genes, residing on chromosomes 16, 6 and 12, respectively (Swan et al., 1979; D'Eustachio

et al., 1980, 1981). In adult mice, λ chains form only about 5% of the total serum Ig light chains and are much less heterogeneous than the κ light chains and heavy chains.

The variable regions of the Ig heavy and light chains each consist of a framework of a relatively conserved amino acid sequence interrupted by three regions of highly variable amino acid sequence. These hypervariable regions interact to form the antigen-contact site of the antibody molecule and thus are referred to as complementarity-determining regions (CDR) (Kabat, 1982). The heavy chain variable region is encoded by the variable (V_H), diversity (D) and joining (J_H) gene segments. Light chain variable regions are encoded by V_L and J_L gene segments only. The germline V_H and V_L gene segments each encode two CDR; the third (CD3) arises from the junctional region when the component gene segments are joined. In the mouse, for the λ light chain two V_L gene segments and four distinct J-C complexes are found (Selsing et al., 1989), while for the κ light chain approximately 300 V_L gene segments, four J_L gene segments and one constant region gene has been found (Zachau, 1989). At the heavy chain locus 4 J_H, 13 D and 200 - 1000 V_H gene segments are found (Figure 1) (Rathbun et al., 1989). The number of V_H gene segments is still debated.

The variable region of the κ light chain can be divided into 18 different groups based on N-terminal amino acid sequence comparisons through the first invariant Trp (Potter et al., 1982). Analysis of restriction fragment length polymorphisms (RFLP) using DNA probes of 14 of the 18 groups in different recombinant mouse strains predicts the following order on chromosome 6: centromer - V11 - V24 - V9-26 - (V9-V1) - V12,13 - (V4-V8-V10-V19) - (V28-Rn75.6) - V23 - V21 - J_κ - C_κ. The gene orders within parentheses have not been determined yet (D'Hoostelaere, 1988).

V_H gene segments have been divided into nine families on the basis of relatedness at the nucleotide sequence level (Brodeur and Riblet, 1984; Winter et al., 1985). Recently three new V_H gene families have been postulated, bringing the total at this moment on twelve V_H gene families (Kofler, 1988; Reininger et al., 1988; Pennel et al., 1989). The V_H genes within a family are highly homologous with more than 80% sequence identity, whereas the degree of homology between members of different families ranges from 50% to 70%. Individual V_H gene families have been estimated to contain from two members in the X24 family to 60 or more members in the J558 family, based on Southern blotting analyses of genomic DNA (Brodeur and Riblet, 1984). Solution hybridization have indicated that the J558 family may involve at least 500 members (Livant et al., 1986). This discrepancy may be due to the presence of many different but related J558 containing fragments in a single Southern band.

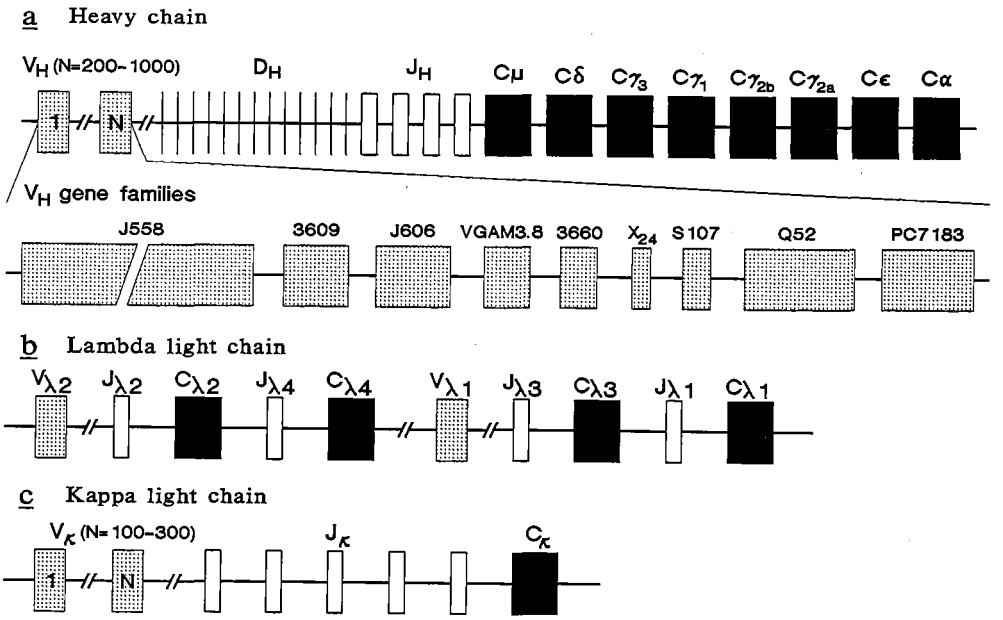

Figure 1 Organization of mouse heavy and light chain Ig genes. For the heavy chain the chromosomal order of the V_H gene families are also shown. This order is based on the most 3' number of each family, since some dispersion and interspersion between V_H gene families has been found. The complexity of each V_H gene family is indicated by the size of the dotted areas. The size of the J558 V_H gene family is still debated (see text).

The V_H gene families are probably organized in overlapping clusters. The various families are located from 5' to 3' in the order J558-3609-J606-VGAM3.8-3660-X24-S107-Q52-PC7183-D_H based on the most recent deletion mapping experiments (Brodeur et al., 1988). This order is based on the most 3' member of each family. S107, 3660 and VGAM3.8 are very dispersed and PC7183 and Q52 are located intermingled on the chromosome. Furthermore some overlap between 3609 and J558 has been found (Brodeur et al., 1988). The recently discovered V_H gene family V_H10 is known to be located 5' of Q52 and PC7183. V_H11 has recently been mapped between X_{24} and 3660 and V_H12 between J606 and VGAM3.8 (Pennel et al., 1989).

V, D and J gene segments are joined by site-specific recombination (reviewed by Tonegawa, 1983 and Alt et al., 1987) (Figure 2). The gene segments that are involved in the rearrangement process are flanked by specific recognition sequences.

These recognition sequences consist of a palindromic heptamer sequence 5'-CACAGTG-3' and a characteristic AT-rich nonamer sequence that is related to the sequence 5'-ACAAAAACC-3'. These two sequences are separated by 12 or 23 non-conserved nucleotides. Joining only takes place if the spacer sequence in one recognition sequence is 12 nucleotides and that of the second 23 (the '12/23 joining rule'). In the lambda light chain gene cluster the Vλ is followed by a 23-bp-spacer, while 5' of Jλ a 12-bp-spacer has been found; for the kappa light chain the situation is just the other way around. Thus V and J light chain gene segments can directly be joined. In the heavy chain gene cluster directly 3' of the V_H gene segments and 5' of the J_H gene segments a 23-bp-spacer can be found. The D gene segments have 12-bp-spacers at both sites. During the generation of a complete Ig heavy chain gene, first a D gene segment is joined to a J_H gene segment and next a V_H gene segment is joined to the D-J_H segment. Sometimes an additional D-J_H can be formed between more 5' D and more 3' J_H gene segments than the one used in the first recombination. Furthermore, recently replacement of V_H gene segments of already rearranged H chain genes (V_H-D-J_H) with that of an upstream germline V_H gene segment has been found

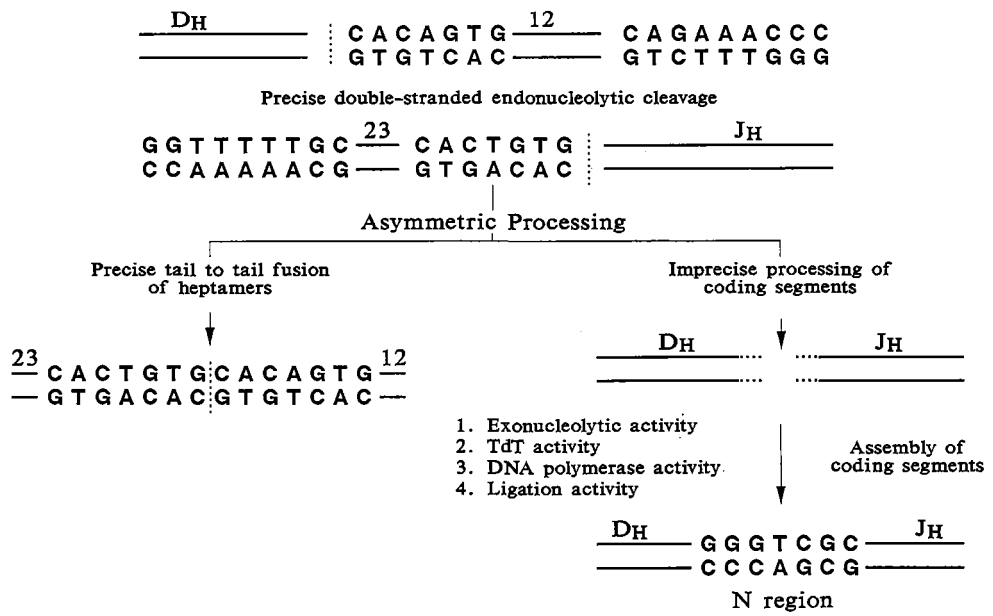

Figure 2 A 'multi-step, nonreciprocal recombination' model for the rearrangement events that are involved in the joining of variable, diversity and joining segments (see text for explanation).

(Reth et al., 1986). A common "Ig-recombinase" is thought to be responsible for this mechanism, since the rearrangement of the light and heavy chain genes and also of the different gene segments coding for the antigen receptors of T cells use the same recognition signals. This is supported by recent data on the genetic defect in murine severe combined immunodeficiency disease (SCID). In SCID mice the Ig-recombinase is defective and not able to allow proper rearrangements, resulting in an absence of functional B and T cells (Malynn et al., 1988). Different models have been proposed for these rearrangements. The currently mostly favoured model (Fig. 2) is the "multi-step, nonreciprocal recombination" model (Alt et al., 1987). In this model the heptamer-spacer-nonamers will combine and this will bring the joining elements adjacent to each other. Subsequently, the two heptamers are precisely ligated (back to back), leading either to the deletion of the intervening DNA as a circle or to its inversion, depending on the chromosomal orientation of the involved segments. Joining of the coding elements occur in a separate step. When the DNA strands are cut, exonucleases may remove some of the terminal nucleotides at both sides. In addition, random nucleotides (N-insertions) can be added to the 3' termini. This is thought to be achieved by the enzyme terminal deoxynucleotidyl transferase (TdT), which is found at the early stages of B (and T) cell development. This leads to imprecise joining of the coding elements which further diversify the specificity repertoire. D segments can be read in different reading frames without introducing stop codons. Furthermore, recently it was shown that both strands of D-coding DNA can be used for producing V-D-J joining (Meek et al., 1989).

Another possible mechanism for Ig gene rearrangement is sister chromatid exchange, but thus far no direct evidence for such a mechanism has been shown. In chickens gene conversion has been shown to be an important mechanism for λ-light chain diversity. In this case only one functional Vλ gene is known, next to approximately 25 Vλ pseudogenes (Reynaud et al., 1987). During λ light chain gene rearrangement, parts of the pseudogenes are integrated into the already rearranged functional Vλ gene by gene conversion. To date this mechanism has not been shown in mammals. Furthermore a process of somatic hypermutation acts upon fully rearranged Ig genes and leads to further diversification of the Ig genes. The molecular basis for the diversification of the B cell repertoire is outlined in Table 1 and this leads to estimates of the potential repertoire of over 10^9 different antigen binding sites.

Table 1	Genetic mechanisms which contribute to the diversity of antibody specificities
1.	Many different germline gene segments (200-1000 V_H, 13D, 4J_H, 2Vλ , 4Jλ , 100-300 Vκ , 5Jκ gene segments).
2.	Combinatorial assortment of the multiple V, D and J gene segments.
3.	Junctional variability by imprecise joining.
4.	Combination of heavy and light chains in the complete antibody molecule.
5.	Somatic hypermutation.

1.3.2. Regulation of expression

All germline V_H genes carry upstream transcriptional promoter elements. Only the promoter of the assembled V_H gene is activated by a tissue-specific enhancer element found in the intron between the J_H gene cluster and the most proximal C_H gene. Sometimes germline V_H and V_L or incomplete D-J_H-chains are transcribed (Yancopoulos and Alt, 1985). These products might play a regulatory role in the establishment of the repertoire.

Transcription of variable and constant parts of both heavy and light chain genes is regulated by RNA processing: post-transcriptional processing mechanisms are responsible for the differential expression of the membrane-bound and the secreted form of the C_H gene products (reviewed in Guise et al., 1989). The Cμ constant region gene, which is the first C_H gene expressed during development, can be replaced by downstream constant region genes during the maturation of a B cell (reviewed in Honjo et al., 1989). This class-switch process allows clonally derived B-lineage cells to maintain the same variable region specificity in association with different heavy chain constant regions determining different effector functions. Recent evidence shows that T-cell derived interleukins play an important role in the regulation of isotype expression (Lee et al., 1986; Snapper and Paul, 1987; Tonkonogy et al., 1989). After generation of a complete Ig gene a B cell produces one type of Ig molecules with unique variable regions and only one type of light chain (κ or λ). This phenomenon is called allelic and isotypic exclusion (Alt et al., 1981). This is probably due to negative feedback mechanisms which are thought to be performed by the protein products. During B cell

development, first the heavy chain genes are rearranged and the membrane form of the heavy chain protein is thought to turn off further heavy chain gene rearrangements and to start light chain gene rearrangements (Storb et al., 1986). From the light chain gene clusters first the κ chain genes are rearranged (Coleclough et al., 1981). It seems that when these κ gene rearrangements are unsuccessful on both alleles the λ chain genes are rearranged, although this timing is not as precise as between heavy and light chain genes (Gollahon et al., 1988).

1.4. Regulation of specificity repertoire

1.4.1. Discrimination of self and nonself

One of the most important characteristics of the immune system is its ability to discriminate between self and nonself. For an effective immune system the lymphocytes have to be tolerant to self and effectively react towards non-self antigens. In recent years it has become clear, mainly by studies in transgenic mice, that for T cells one important way of tolerance induction is clonal deletion of self-reactive T lymphocytes within the thymus (Kisielow et al., 1988). However, some additional suppressive mechanisms in the periphery are still necessary. For B cells no such mechanism for tolerance induction in a specialized organ is known. In double transgenic mice which express the genes for a 'neo-self antigen', hen egg lysozyme, and a high affinity anti-lysozyme antibody, most anti-lysozyme B cells did not undergo clonal deletion, but were not able to secrete high affinity anti-lysozyme antibody (Goodnow et al., 1988). This points towards another mechanism to functionally silence high-affinity self-reactive B lymphocytes.

B cells with the capacity of secreting autoantibodies have been shown to be normal components of the immune system. Normal human serum contains antibodies against neurofilaments, tubulin, actin, transferrin, thyroglobulin etc. (Guilbert et al., 1982). These antibodies are usually of the IgM class and of relatively low affinity. Early in ontogeny a high frequency of B cells can be found which can bind to multiple antigens, among which autoantigens. Such B cells have been called 'multireactive B cells' (Dighiero et al., 1985). The antibodies produced by many of such B cells can also recognize different antibody combining sites or determinants specific to Ig variable regions of other B cells (e.g. idiotypes). They are called "highly connective" antibodies and they may participate in an idiotypic network (Holmberg et al., 1984). The idiotype network will be discussed in detail later.

In pre-B cell lines that differentiate in culture and hybridomas derived from fetal liver and neonatal spleen a preferential utilization of the V_H gene segments which are

most proximate to the genes coding for the constant part of the heavy chain has been found (Yancopoulos et al., 1984; Perlmutter et al., 1985; Holmberg, 1987). The same bias has been found in pre-B cell lines derived from adult bone marrow, although not as striking as during ontogeny (Yancopoulos et al., 1984). This is in contrast with hybridomas, B cell cultures and LPS-stimulated B cells derived from adult spleen where the usage of V_H gene family is more related to V_H gene family size (Dildrop et al., 1985; Wu and Paige, 1986; Schulze and Kelsoe, 1987). The selective forces that are responsible for the difference between the primary B cell repertoire found during early B cell differentiation both in ontogeny and in the adult bone marrow and the repertoire of adult splenic (peripheral) B cells are still unknown. That some selection is going on is consistent with the notion that only 10% of the early B cell precursors in the bone marrow gives rise to mature adult splenic B cells. This can partially be explained by the intracellular constraints of the recombinatorial processes of Ig assembly, like abortive rearrangements and unappropriate heavy and light chain pairing. But also selection by external forces should be considered. Cellular selection would act on primary B cells and involve environmental antigens and/or T cells and other endogenous antigens acting on surface antibody receptors.

1.4.2. Antigen selection

The Ig at the surface of newly formed B cells act as an antigen receptor. After contact with the appropriate antigen, B cells will proliferate and differentiate into clones of Ig-secreting cells (reviewed in Rajewsky et al., 1987). During the course of a humoral immune response different differentiation pathways can be distinguished. After the first contact with antigen a primary response is seen which is characterized by predominant IgM production and, dependent on the type and dose of antigen, somewhat later IgG production. At the same time, by a so far unknown mechanism, some B cells are induced to become memory B cells. After the second and subsequent encounters with the same antigen a secondary type response develops. Such responses to T dependent antigens are characterized by a more rapid induction of Ig production of mainly the IgG class and a higher production of Ig with higher affinity for the antigen.

The selection of B cells producing high affinity antibodies by antigen during the course of the immune response has been studied by fusion of antibody secreting cells with a non-secreting myeloma cell line (so called hybridomas) (Köhler and Milstein, 1975). It was shown by the generation of hybridomas after primary, secondary and tertiairy antigen administration that the higher affinity was caused by somatic mutations in the genes coding for the variable heavy and light chain regions (Bothwell et al., 1981; Berek et al., 1985; Siekevitz et al., 1987). These somatic mutations are the result of a B-cell specific somatic hypermutation process which is calculated to

introduce 1 x 10^{-3} somatic mutations per base pair per generation (McKean et al., 1984). B cells with somatic mutations leading to higher affinity antibodies are selected for by the antigen. If an optimum affinity has been reached the somatic hypermutation process is no longer advantageous.

The relationship between the process of somatic mutation and that of the concurrent event of isotype switch is still a matter of speculation. An important argument that the somatic hypermutation process occurs before the isotype switch is that clonally related B cells with (partially) the same somatic mutations that had undergone switching all showed different switch recombination breakpoints (Rajewsky et al., 1987). It even has been proposed that the isotype switch may be the molecular event that terminates the hypermutation process (Rajewsky et al., 1987). This, however, does not seem to be the case, since clonally related IgG antibodies with different somatic mutations and identical class switch regions have also been isolated (Shlomchik et al., 1989).

1.4.3. Network theories

Based on the fact that every Ig molecule has an unique variable region which might be recognized by other antibody molecules, Jerne has postulated the network theory (Jerne, 1974). Each Ig molecule bears a combining site (paratope) that can interact with antigenic determinants (epitopes) of conventional antigens and with other Ig molecules which possess one or more antigenic determinants, defined by the structure of its variable region, termed idiotypic determinants or idiotopes. One of the postulates of the network theory is that Ig molecules should exist which express idiotopes mimicking naturally occurring antigenic determinants (epitopes). Such idiotopes are designated as the internal images of antigens present for example in the external environment of the individual. For each antibody expressing a given paratope and idiotype (set of idiotopes) a complementary antibody must exist possessing a paratope capable of binding to the idiotope of the first one and expressing its own set of idiotopes and so on. Under the assumption that paratope-idiotope interactions both can stimulate and suppress the idiotope-bearing cells, depending for instance on the way of recognition, one can imagine a dynamic equilibrium, even in the absence of exogenous antigen. This total set of interactions is thought to be in equilibrium in the individual and antigen can then be viewed upon as acting to disturb this equilibrium and an immunological reaction as a means to reach a new equilibrium in the system. The network theory has since then been extended to an unifying hypothesis where complementarity of not only Ig, but also the T-cell receptor and all other structures which are available, are involved, in order to establish a homeostasis (Coutinho et al., 1984). The formal existence of an idiotypic network has been shown in neonatal, not

antigen-stimulated B cells (Holmberg et al., 1984) and by immunization with idiotypes in order to produce idiotype-specific antibodies (Kelsoe et al., 1980). The way that anti-idiotypic T cells are able to recognize idiotopes in an MHC restricted manner is still unclear. There are some experiments that show that anti-idiotypic T cells can recognize an antigen-processed Ig heavy chain. Whether the idiotypic network indeed plays a major functional role in vivo in the immunoregulation is still unclear. However, it is possible to manipulate certain immune responses with anti-idiotypic Ig, especially when they are administered early in ontogeny (Kearney and Vakil, 1986).

Multireactive antibodies, which are predominant early in ontogeny, form a 'highly connective' idiotypic network, which may play a role in the early establishment of the specificity repertoire which is in homeostasis with the self antigens.

Network interactions are not only dependent on the quality of recognition between idiotypes and anti-idiotypic antibodies, but also on the number of cells bearing such receptors within a diverse population. An attractive model for this regulation at the level of cell populations is presented by the 'balance of growth hypothesis' of Grossman (Grossman, 1984). In this model it has been proposed that the development of a clone is greatly affected by factors that determine the balance between self-renewal and differentiation. Major factors are both exogenous and endogenous antigens. The model predicts that there are only quantitative differences between the immune response towards endogenous and exogenous antigens.

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CHAPTER 2

INTRODUCTION TO THE EXPERIMENTAL WORK

The exogenous antigenic environment plays an important role in the selection of the specificity repertoire of B cells. The influence of antigens on the different stages of B cell development is difficult to assess, because from birth on the organism is always exposed to exogenous antigens. In order to study the role of exogenous antigenic stimulation on the development of the B cell specificity repertoire, we have used mice which were deprived of exogenous antigenic stimulation as far as possible.

The best known model for antigen deprivation is to keep mice under germfree (GF) conditions and to feed them a chemically defined (CD), ultrafiltered diet of low molecular weight components (< 10.000 D) in order to avoid antigenic stimulation by macromolecules from the food. Mice can be kept under such conditions for more than ten generations. The young first suckle maternal milk and after weaning change to the CD diet. Such mice show a normal physiology, as far as reproduction and mortality are concerned. Their growth rate appears somewhat slower shortly after weaning, but thereafter they gain weight more rapidly than control mice. This is probably due to the problem of learning to eat the soluble diet from overhanging bottles. Some male mice seem to suffer from colonic impaction of cecally formed trichobezoars. These losses were judged to be unrelated to nutritional deficiency. No overt signs of nutritional deficiency developed in mice which were fed CD diet up to 18 months of age. We have compared the specificity repertoires of the B cells of such GF-CD mice with that of control mice which were kept under conventional stable conditions and fed a natural ingredient diet (CV-NI).

In Chapter 3 we studied the development of the actual specificity repertoire in young of GF-CD and CV-NI BALB/c mice from birth on up till the young adult age. To this end total numbers of background Ig-secreting cells of different isotypes and the frequencies of antigen-specific IgM-secreting cells were determined in plaque assays.

Determination of the specificity repertoire of 'background' Ig-secreting cells in plaque-assays can only be determined accurately in plaque-assays for IgM-secreting cells. Because the greatest numerical changes in the number of Ig-secreting cells due to exogenous antigenic stimulation occur in the IgA- and IgG- (sub)classes, we used the ELISA plaque assay, which is appropriate to determine numbers of antigen-specific Ig-secreting cells of all isotypes. By using DNP27-BSA and different monoclonal anti-idiotypic antibodies as antigens, the total numbers and the frequencies of antigen-specific IgM-, IgG- and IgA-secreting cells were determined in GF-CD and CV-NI BALB/c mice (Chapter 4).

In Chapter 5 we compared the serum Ig levels of GF-CD and CV-NI mice using a sensitive sandwich-ELISA-system. Furthermore we investigated the occurrence of natural antibodies against several carbohydrate antigens.

During pregnancy many immunological parameters change, even in syngeneically mated mice. During pregnancy the spleen and lymph nodes are enlarged and the thymus is reduced in size. The reasons for these changes are unclear. In order to investigate to what extent these changes are caused by external antigens, or by internal (e.g. fetal or hormonal) influences, we investigated the B cell repertoire in pregnant GF-CD mice. To this end the numbers of IgM-, IgG- and IgA-secreting cells in the spleen, bone marrow and mesenteric lymph nodes and the frequencies of antigen-specific IgM-secreting cells in the spleen and bone marrow of pregnant GF-CD and CV-NI BALB/c mice were compared to the appropriate virgin controls (Chapter 6).

The available specificity repertoire of GF-CD mice was studied by producing a large number of hybridomas from LPS-reactive splenic B cells. These hybridomas were studied for the usage of the different V_H gene families with an RNA blot assay and compared with a hybridoma collection that was made from the splenic B cells of 5 day old neonatal conventional (CV-NEO) mice (Chapter 7).

In Chapter 8 we screened both hybridoma collections for antigen specificities against a large panel of exogenous and endogenous antigens. As a source for natural exogenous antigens 36 different bacterium strains were tested and nine different viruses, while as endogenous antigens were used frozen tissue sections of stomach, liver and kidney, the Hep-2 cell line and the anti-idiotypic antibodies Ac38 and Ac146.

In Chapter 9, the General discussion, we compare the data emerging from our studies with those in the literature and present a hypothesis on the regulation of the specificity repertoire by exogenous antigens.

CHAPTER 3

EARLY DEVELOPMENT OF Ig-SECRETING CELLS IN YOUNG OF GERM-FREE BALB/c MICE FED A CHEMICALLY DEFINED ULTRAFILTERED DIET

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Early Development of Ig-Secreting Cells in Young of Germ-Free BALB/c Mice Fed a Chemically Defined Ultrafiltered Diet

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The influence of antigenic stimulation on the early development of the "spontaneously" occurring ("background") IgM-, IgG-, and IgA-secreting cells has been studied in mice. To evaluate the effect of such exogenous stimulation by an evolving microbial microflora, the young of BALB/c mice that were kept under germ-free conditions and fed a low molecular weight chemically defined synthetic diet (GF-CD) were compared with the young of conventional BALB/c mice fed natural ingredients (CV-NI). The young were first suckling maternal milk and between Days 15 and 18 changed to the same diet as their parents. Background Ig-secreting cells in the spleen were enumerated in the protein A plaque assay. The specificity repertoire of the IgM-secreting cells was determined with plaque assays specific for sheep red blood cells (SRBC) that were haptenized with different concentrations of nitroiodophenyl (NIP), 4-hydroxy-3.5-dinitrophenyl (NNP), and 2,4,6-trinitrophenyl (TNP). The results show that during the first few weeks of life the numbers of background IgM-, IgG-, and IgA-secreting cells in the spleen develop faster in CV-NI mice than in GF-CD mice. At 4 weeks of age equal numbers of IgM- and IgG-secreting cells were found in both groups of mice, but the number of IgA-secreting cells remained reduced in GF-CD mice during the whole period of observation. The frequencies of IgM-secreting cells specific for the differently haptenized SRBC were the same in both groups of mice during the observation period of 10 weeks. This suggests that the ontogenetic appearance of IgM-, IgG-, and IgA-secreting cells in the spleen, and the specificity repertoire of the IgM-secreting cells, as far as was tested in our panel, is independent of exogenous antigenic and/or mitogenic stimulation. However, during neonatal development the rate of development of the background Ig synthesis is enhanced by environmental antigenic stimulation.

INTRODUCTION

The development of the B-cell system during ontogeny has been studied extensively (reviewed in (1, 2)). In mice, surface immunoglobulin (Ig)-positive (B) cells were detected first in liver, spleen, and bone marrow (BM) at Day 17 of gestation (3). While in the liver the number of B cells rapidly decreases shortly after birth, in the spleen and BM their number expands until they reach plateau levels between 1 and 4 months of age. These changes take place while the animal first is confronting exogenous antigens. It is well documented that during this critical period of B-cell develop-

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ment the specificity repertoire is influenced by many regulatory mechanisms. Neonatal mice show a different pattern of reactivity against a number of antigens, e.g., dextran (4) and phosphorylcholine (5), as compared to adults. Furthermore, during the first period of life, the B-cell system is highly susceptible to tolerization (6). Another example of the importance of this period of development is the sequential appearance of reactivity to thymus-independent type 1 and thymus-dependent and thymus-independent type 2 antigens (7).

The influence of the antigenic environment on the establishment of the B-cell repertoire is not well understood. To investigate this we used germ-free BALB/c mice given an ultrafiltered, chemically defined, low molecular weight synthetic diet (GF-CD) and compared them with conventionally reared BALB/c mice fed a natural ingredient diet (CV-NI).

We have determined the number of spontaneously occurring ("background") Ig-secreting cells in the spleen of both GF-CD and CV-NI mice from birth up to Week 10 with the use of the protein A plaque assay. These mice initially were fed maternal milk and later the same diet as their parents. Furthermore, we have looked at the specificity repertoire of the IgM-secreting cells with the use of plaque assays specific for differently haptenized sheep red blood cells (SRBC). These studies reveal that there is a considerable influence of exogenous antigens on the rate of the development, but not on the establishment of the specificity repertoire of the background IgM-secreting cells.

MATERIALS AND METHODS

Mice. BALB/cAnN mice, that were obtained from the University of Wisconsin, and in 1983 had been obtained from the National Institutes of Health, were reared and maintained in the Lobund Laboratory, University of Notre Dame (Notre Dame, IN) either germ-free and fed chemically defined ultrafiltered "antigen-free" diet L489-E14Se and LADEK 69E6 (GF-CD) as described in detail (8), or specific pathogen-free (SPF) and fed natural ingredient L-485. Other SPF BALB/c mice were obtained from OLAC 1976, Ltd. (Blackthorn, England) and Bomholtgard, Ltd. (Ry, Denmark) and were reared and maintained under nonbarrier conventional stable conditions and fed conventional, nonautoclaved diet AMII (Hope Farm, Woerden, The Netherlands) at the Department of Cell Biology, Immunology, and Genetics, Rotterdam, The Netherlands (CV-NI). All animal stocks originally had been obtained from the Jackson Laboratory and were routinely checked for genetic origin by the supplier. All breeding experiments were performed within 6 months after the obtainance of the animals. The conventional BALB/c mice were free of ectoparasites and were checked routinely for their microbiological status. During the experiment *Pasteurella pneumotropica* was found in the nasopharynx of the CV animals.

For mating purposes, one BALB/c female mouse was housed with a syngeneic BALB/c male mouse. The young had both maternal milk and diet available up to weaning at Day 25 and are assumed to have started consuming their parents' diet at Days 16 to 19 while continuing to suckle until weaning. They were tested from Day 1 to Day 28 of age. The day of birth was considered to be Day 1. Furthermore, 10-week-old BALB/c mice were used as adult control mice and referred to as 70-day-old mice in the figures. The data obtained from male and female mice were pooled. All mice were used within 1 hr of removal from the colony. It should be mentioned

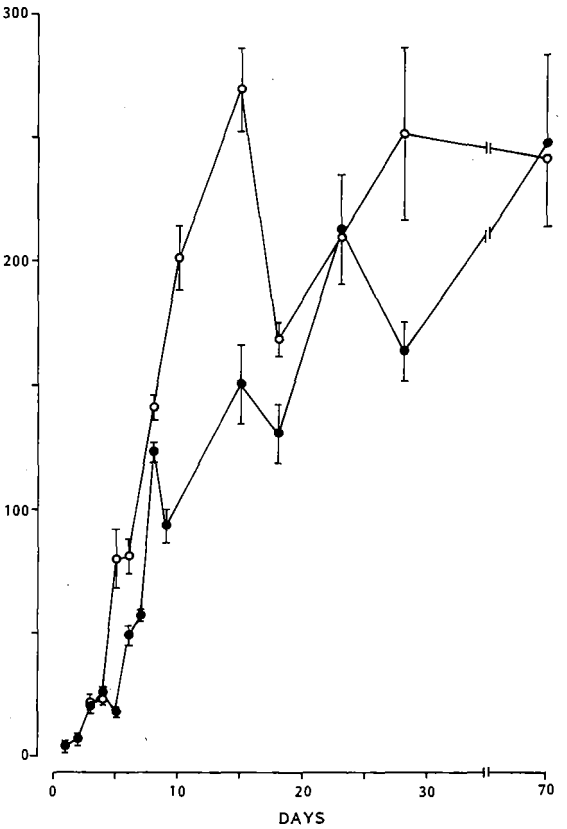

FIG. 1. Numbers of nucleated cells in the spleen of GF-CD (O) and CV-NI (●) BALB/c mice at various stages of development. Figures represent the arithmetic mean of two to eight mice ± SE.

that all mice called "germ-free" carry a latent leukemogenic virus that can be activated by repeated low doses of radiation (9, 10).

Preparation of lymphoid cell suspensions. Spleen, BM, and mesenteric lymph nodes (MLN) cell suspensions were prepared as described by Benner *et al.* (11). The total numbers of nucleated cells were counted with a Coulter Counter, Model BZI (Coulter Electronics, Harpenden, Berks, England).

Plaque assays for Ig- and antibody-secreting cells. The target cells for the protein A plaque assay and the antigen-specific plaque assays were prepared, and the plaque assays were performed as has been described earlier (12, 13). Briefly, nitroiodophenyl (NIP), 4-hydroxy-3,5-dinitrophenyl (NNP), and 2,4,6-trinitrophenyl (TNP) were coupled to SRBC with 4 or 0.4, 2 or 0.2, or 30 mg of the hapten per milliliter of washed and packed SRBC, respectively. These haptenized SRBC are referred to as NIP4-SRBC, NIPO.4-SRBC, NNP2-SRBC, NNP0.2-SRBC, and TNP30-SRBC.

Antisera. The specificity of all antisera used was confirmed in the protein A plaque assay with the appropriate myeloma and hybridoma cell suspensions.

Calculation of the total number of Ig-secreting cells and frequency determination of background antigen-specific IgM-secreting cells. The total number of Ig-secreting cells of a given class per organ was calculated by using the number of Ig-secreting cells in the protein A plaque assay and the total cell yield per organ. The frequencies of

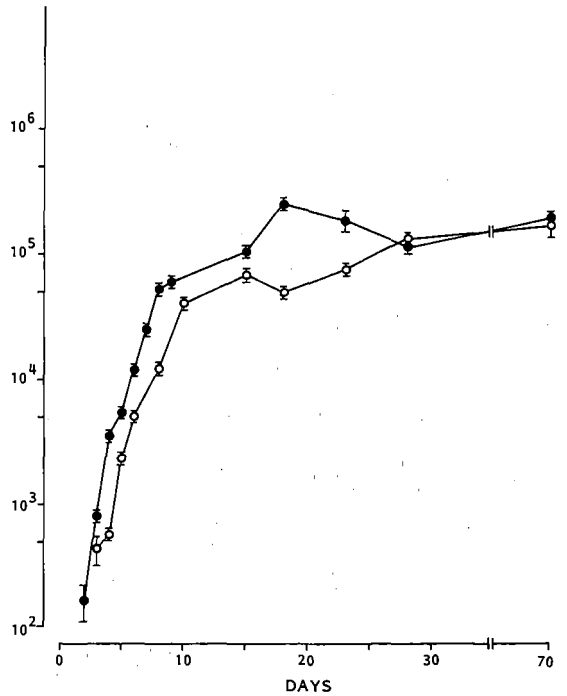

FIG. 2. Numbers of IgM-secreting cells in the spleen of GF-CD (○) and CV-NI (●) BALB/c mice at various stages of development. Figures represent the arithmetic mean of two to eight mice ± SE.

background antigen-specific IgM-secreting cells in the spleen were determined in the appropriate plaque assays and calculated as the ratio of the specific IgM antibody-secreting cells to the total number of IgM-secreting cells.

Statistical analysis. The differences between the experimental groups of mice were evaluated with the Student *t* test and the Mann–Whitney test.

RESULTS

Number of Nucleated Cells in the Spleen

The number of nucleated cells in the spleen of neonatal GF-CD and CV-NI BALB/c mice was determined at various intervals after birth (Fig. 1). In both groups the cell number rapidly increased from a few million cells at birth up to approximately 250×10^6 and 150×10^6 at Day 15 in the GF-CD and CV-NI mice, respectively. Between Day 15 and Day 18, in both groups of mice the number of nucleated cells decreased and afterward increased gradually to a plateau level of about 250×10^6 cells per spleen in adult mice.

The decrease between Days 15 and 18 was faster in GF-CD mice than in CV-NI mice.

Development of Background Ig-Secreting Cells

By means of the protein A plaque assay the total numbers of "background" IgM-, IgG-, and IgA-secreting cells were determined in the spleen of GF-CD and CV-NI mice from birth up to Day 28 and in 70-day-old mice.

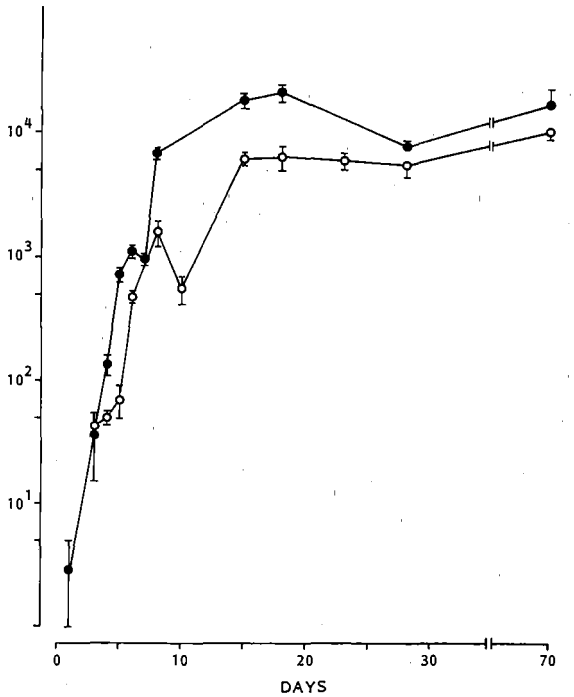

FIG. 3. Numbers of IgG-secreting cells in the spleen of GF-CD (○) and CV-NI (●) BALB/c mice at various stages of development. Figures represent the arithmetic mean of two to eight mice ± SE.

The number of IgM-secreting cells increased exponentially during the first 10 days of life in both groups of mice (Fig. 2). In the CV-NI mice, however, the absolute numbers were significantly higher ($P < 0.05$) than in the GF-CD mice in this stage of development. Between Day 10 and Day 23 the number of IgM-secreting cells increased more gradually, in both groups, but remained higher in the CV-NI mice than in the GF-CD mice. After Day 23 both groups of mice had the same number of approximately 170,000 IgM plaque-forming cells (PFC) per spleen.

The number of IgG-secreting cells in the spleen also increased exponentially in both groups of mice during the first 8 days of life (Fig. 3). Except for Day 3 the absolute numbers of the CV-NI mice were significantly higher than in the GF-CD mice ($P < 0.05$). Thereafter the CV-NI mice developed more IgG-secreting cells than the GF-CD mice, although the difference became smaller at 4 weeks of age.

The numbers of background IgA-secreting cells greatly differed between the two groups of mice (Fig. 4). During the first 4 days after birth the CV-NI mice showed a relatively high number of IgA-secreting cells and, after a short decline, grew exponentially up to more than 10,000 cells per spleen around Day 10. The numbers in GF-CD mice, at almost all time points, were lower than in age-matched CV-NI mice. This was still so at 70 days after birth.

Background Ig-Secreting Cells per 10^6 Nucleated Cells

Because the spleen consists of several different cell populations that show great numerical changes during the first weeks of life, it was of interest to calculate the

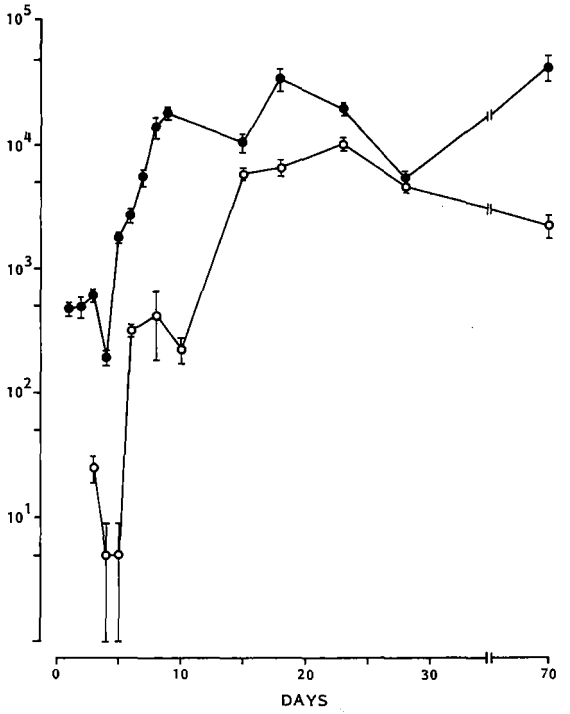

FIG. 4. Numbers of IgA-secreting cells in the spleen of GF-CD (O) and CV-NI (●) BALB/c mice at various stages of development. Figures represent the arithmetic mean of two to eight mice ± SE.

relative increase of background Ig-secreting cells during this period. Therefore we summed up the numbers of IgM-, IgG-, and IgA-secreting cells per spleen and divided it by the number of nucleated cells of the spleen of the same mouse. Although the GF-CD mice had more nucleated cells per spleen than the CV-NI animals (Fig. 1), they had a smaller number of Ig-secreting cells per spleen (Figs. 2, 3, and 4). This resulted in great differences in the number of background Ig-secreting cells per 10^6 nucleated cells between GF-CD and CV-NI mice (Fig. 5). This relative number of Ig-secreting cells reached a peak number of approximately 2500 at Day 18 and afterward declined to about 1000 Ig-secreting cells per 10^6 nucleated cells in adult CV-NI mice. The GF-CD mice showed a much slower increase and reached a plateau level of 1000 Ig-secreting cells per 10^6 nucleated cells at the young adult age. Thus in the young adult mice the relative number of Ig-secreting cells is the same in both groups.

We also investigated a group of conventional mice that directly originated from the GF-CD mice and were kept under very clean (laminar flow), stable conditions and that were fed autoclaved natural ingredient diet. They showed no significant difference in the number of IgM-secreting cells during neonatal development as compared to the GF-CD mice, but the number of IgG- and IgA-secreting cells were significantly higher at several time points during the first 10 days, although their numbers were not as high as in the CV-NI mice studied in detail (data not shown). So there seems to be a direct relationship between the rate of neonatal development of "background" Ig-synthesis and the exogenous antigenic load.

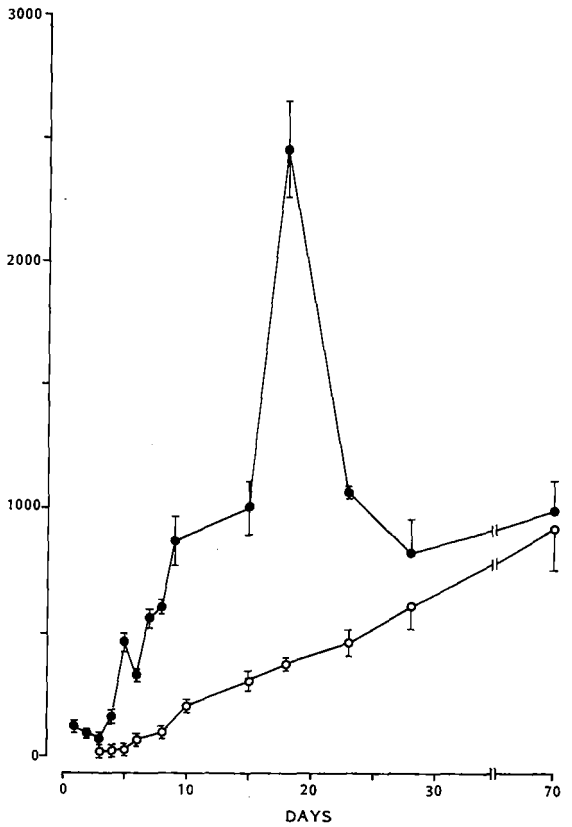

FIG. 5. Numbers of Ig-secreting cells per 10^6 nucleated spleen cells of GF-CD (O) and CV-NI (●) BALB/c mice. The numbers of Ig-secreting cells plotted are the sum of the numbers of IgM-, IgG-, and IgA-secreting cells determined in the appropriate protein A plaque assays. The same mice were used as for the experiments shown in Figs. 1–4.

Frequencies of Background Antigen-Specific IgM-Secreting Cells

The frequencies of background antigen-specific IgM-secreting cells were determined in the spleen of neonatal CV-NI and GF-CD mice by employing antigen-specific plaque assays with the use of five different types of target cells. By comparing their number with the number of all IgM-secreting cells together as judged by the protein A plaque assay, the relative frequency of the defined specificities was calculated. The results given in Table 1 show that the frequency of IgM-secreting cells specific for the differently haptenized SRBC did not change during neonatal development in either the GF-CD or the CV-NI mice. The only exception was the frequency of NNP0.2 SRBC-specific IgM-secreting cells in the GF-CD mice at 70 days, which was significantly lower ($P < 0.01$).

DISCUSSION

In this study we investigated the role that exogenous antigens might play in the neonatal development of the B-cell repertoire as it is expressed by "spontaneously"

TABLE 1

Relative Frequencies of Background IgM-Secreting Cells Specific for NIP4-SRBC, NIP0.4-SRBC, NNP2-SRBC, NNP0.2-SRBC, and TNP30-SRBC in the Spleen of Conventional BALB/c Mice Fed Natural Ingredient (CV-NI) and Germ-Free BALB/c Mice Fed Chemically Defined Diet (GF-CD) during Neonatal Development

Mice	Antigen	Day 5	Day 8	Day 15	Day 25	Day 70
CV-NI	NIP4-SRBC	1 in 16 (15–17)[a]	1 in 20 (19–21)	1 in 13 (13–14)	1 in 15 (13–18)	1 in 20 (19–22)
	NIP0.4-SRBC	1 in 214 (186–245)	1 in 123 (113–134)	1 in 133 (117–151)	1 in 103 (81–130)	1 in 169 (140–240)
	NNP2-SRBC	1 in 50 (46–54)	1 in 43 (40–46)	1 in 34 (32–36)	1 in 30 (26–34)	1 in 36 (33–40)
	NNP0.2-SRBC	1 in 321 (253–408)	1 in 213 (193–235)	1 in 417 (321–542)	1 in 256 (218–302)	1 in 1087 (901–1310)
	TNP30-SRBC	1 in 94 (90–98)	1 in 89 (84–94)	1 in 48 (44–52)	1 in 37 (32–44)	1 in 117 (106–129)
GF-CD	NIP4-SRBC	1 in 24 (21–26)	1 in 16 (15–17)	1 in 21 (20–22)	1 in 28 (24–32)	1 in 37 (31–44)
	NIP0.4-SRBC	1 in 80 (67–95)	1 in 56 (52–60)	1 in 52 (46–58)	1 in 70 (57–85)	1 in 159 (137–185)
	NNP2-SRBC	1 in 26 (24–29)	1 in 17 (15–18)	1 in 24 (23–25)	1 in 30 (27–33)	1 in 55 (46–66)
	NNP0.2-SRBC	1 in 232 (186–289)	1 in 231 (206–259)	1 in 159 (137–184)	1 in 190 (154–235)	1 in 202 (169–240)
	TNP30-SRBC	1 in 96 (75–122)	1 in 77 (70–85)	1 in 78 (75–81)	1 in 106 (80–144)	1 in 217 (202–235)

[a] Data represent the mean ratios of specific IgM-antibody-secreting cells to the total numbers of IgM-secreting cells as determined in the protein A–plaque assay. The same samples were used to determine the total numbers of IgM-, IgG-, and IgA-secreting cells (Figs. 2–4). The geometric mean ± 1 SEM of four to eight individually tested mice has been calculated.

occurring ("background") Ig-secreting cells in not intentionally immunized mice. We used germ-free mice nursed by females that were given a chemically defined, ultrafiltered, low molecular weight synthetic diet for successive generations so that stimulation of the immune system by exogenous antigens was excluded as far as technically can be achieved at this moment (8). The young were weaned to their mother's diet. The results show that such "antigen-free" mice already have Ig-secreting cells at birth. Apparently, endogenous stimulation accounts for activation and terminal differentiation of the B cells involved.

The numbers IgM-, IgG-, and IgA-secreting cells increased more rapidly in CV-NI mice than in GF-CD mice. A comparable neonatal development in the number of background Ig-secreting cells has been found in conventional C_3H/HeJ mice by Björklund *et al.* (14), although they did not detect IgA-secreting cells before 3 weeks of age, while we found already a detectable number at Day 3. This might be due to the different batches of antiserum used and/or the modifications that we introduced in the protein A plaque assay (15).

Because we have examined only the spleen and do not have insight into the effect of important variables such as cell migration and death rates, we do not know whether the age-related increase of the number of Ig-secreting cells in the spleen is caused by cell migration or local production.

It can be speculated that some antigenic stimulation triggers a nonspecific rise in the activity of the B-cell network, e.g., by nonspecific growth and differentiation factors derived from T cells, specifically activated by endogenous idiotypes. A similar effect of exogenous antigens, and also from nonantigenic stimuli, has been noted on B lymphocyte production in the BM (16). The exogenous antigenic stimulation, however, is not obligatory for the development of the background Ig synthesis in the spleen, because GF-CD mice reached the same adult level of background Ig synthesis as the conventional mice although at a lower rate.

In the two groups of 10-week-old mice we found comparable absolute as well as relative numbers of background Ig-secreting cells in the spleen. But the "path" by

which those values were reached is quite different (cf. Figs. 1 and 5). The absolute number of IgA-secreting cells, however, was clearly higher in the CV-NI mice at 10 weeks of age (Fig. 4). This is in agreement with earlier experiments using adult C_3H/HeCr mice (17), which showed that the background Ig synthesis in the spleen is mainly due to endogenous stimulation. In contrast, the background Ig synthesis in the BM and MLN, especially the IgG and IgA synthesis, seems to be mainly dependent on exogenous stimulation (17). We confirmed these data for BALB/c mice as we also found a 30- to 1000-fold decrease of the number of IgG- and IgA-secreting cells in these organs of GF-CD BALB/c mice (18).

Velardi and Cooper (3) showed by immunofluorescence staining of different populations in the spleen of conventional mice that during the first weeks of life there is an increase not only in the number of B cells, but also of T cells. The number of macrophages as detected with the monoclonal antibody Mac-1 reached their plateau level already around Day 5. Furthermore, it is known that in conventional mice the number of cytoplasmic μ+, sIg− pre-B cells in the spleen increases temporarily around Day 5. These cells, however, disappeared at Day 15 (3, 19). Differences in these non-B-cell populations also might contribute to the increased number of nucleated cells in the spleen of GF-CD mice during the first few weeks of life. In view of these population dynamics an interesting observation is the sudden decrease of the number of nucleated cells in the spleen around Days 15 to 18 in both the CV-NI and the GF-CD mice, which resulted especially in the CV-NI mice in a temporarily increase in the relative number of Ig-secreting cells per million spleen cells (Fig. 5). This was tested many times in different litters, so it cannot be explained by experimental variation. Apart from changes in the number of different populations in the spleen around Day 15 (3), another reason for this might be that the neonatal mice cannot obtain enough nutrients from nursing and must temporarily suffer from some nutrient or water deficiency. The conventional mice which have been fed with natural ingredients show a less clear regression in the number of nucleated cells than the neonatal GF-CD mice which had to learn to drink the soluble synthetic diet from overhead bottles and thus might suffer more. Malnutrition has been shown to influence the body and spleen weights, but as soon as the malnutrition has been ended these parameters normalize again (20).

In order to examine the influence of exogenous antigens on the B-cell specificity repertoire, we determined the frequencies of background antigen-specific IgM-secreting cells. The results show no gross changes in the frequencies of IgM-secreting cells specific for the investigated antigens during early development. Even at Day 5 after birth, while there is a 50 to 100 times lower number of IgM-secreting cells, we found the same frequencies of IgM-secreting cells specific for the antigens tested as in adult mice. Furthermore, the frequencies of these specificities in GF-CD and CV-NI mice were comparable during the whole period of observation. This suggests that as far as the IgM-secreting cells are concerned and as far as was tested in our panel of haptenized SRBC, endogenous stimulation largely determines the IgM-specificity repertoire. This might be caused by idiotypes, e.g., from maternal Ig transferred to the pre- or neonates via the placenta or milk or from any other self antigen (21). Experiments with hybridomas, obtained from 5-day-old unimmunized BALB/c mice, show that natural autoantibodies frequently occur in normal newborn mice (22), which might reflect such endogenous stimulation. Furthermore, during pregnancy the number of background Ig-secreting cells change markedly in both the CV-NI and the GF-CD

mice and this might also influence the B-cell development of the young (18). The constant frequencies of the various specificities among the background Ig-secreting cells are in direct support of Jerne's network theory (23). He proposed that naturally occurring antibodies are responsible for the establishment and maintenance of a complete network including antibodies and T cells. Consequently, T cells also might play a role in the development of the B-cell specificity repertoire (24, 25).

Background Ig-secreting cells reflect the so-called "actual" repertoire of B cells which are stimulated *in vivo* by a variety of endogenous and, in conventional mice, exogenous stimuli (26). This actual repertoire is determined by the so called "available" B-cell repertoire of mature B cells and all regulatory influences of the immune system. The available specificity repertoire of B cells of mature conventional and germ-free mice as determined in the splenic focus assay (27) and by limiting dilution analysis of LPS-reactive B cells (28) does not seem to be very much different.

The frequency of hapten-specific B cells (representing the available specificity repertoire) of conventional neonatal and adult mice (29) also does not seem to differ, although there is a restriction in the heterogeneity of these cells in neonatal mice (30–32). It remains to be established whether the neonatal GF-CD mice have a similar available B-cell repertoire as neonatal CV-NI mice. This is especially of interest in view of the reported sequential acquisition of reactivity to antigens as α-1,3 dextran (4) and thymus-independent derivatives of phosphorylcholine (5).

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CHAPTER 4

THE INFLUENCE OF EXOGENOUS ANTIGENIC STIMULATION ON THE SPECIFICITY REPERTOIRE OF BACKGROUND IMMUNOGLOBULIN-SECRETING CELLS OF DIFFERENT ISOTYPES

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The Influence of Exogenous Antigenic Stimulation on the Specificity Repertoire of Background Immunoglobulin-Secreting Cells of Different Isotypes

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The total number of spontaneously occurring ("background") IgM-, IgG-, and IgA-secreting cells and the frequency of antigen-specific IgM-, IgG-, and IgA-secreting cells were determined in germ-free BALB/c mice fed a chemically defined ultrafiltered diet (GF-CD), in specific pathogen-free BALB/c mice fed an autoclaved natural ingredient diet (SPF-NI), and in conventional BALB/c mice fed nonautoclaved natural ingredients (CV-NI). This was done by means of the ELISA–plaque assay. The results did not show differences among the various groups of mice with regard to the total numbers of IgM-secreting cells in the various lymphoid organs. Also the frequencies of IgM-secreting cells specific for DNP27-BSA and the anti-idiotypic monoclonal antibodies Ac38 and Ac146 did not differ significantly among GF-CD, SPF-NI, and CV-NI mice. GF-CD mice, however, did show substantially decreased numbers of IgG- and IgA-secreting cells in their lymphoid organs. Furthermore, there were striking differences in the frequencies of antigen-specific IgG- and IgA-secreting cells between GF-CD mice and the two other groups of mice. These results indicate that exogenous antigenic stimulation has a great effect on both the total numbers and the specificity repertoires of background IgG- and IgA-secreting cells. Such an influence could not be detected with regard to the background IgM-secreting cells. This suggests two distinct compartments of background Ig-secreting cells: a very stable, endogenously regulated compartment consisting mainly of IgM-secreting cells, and another compartment, consisting mainly of IgG- and IgA-secreting cells, whose numbers and specificity repertoire appeared to be influenced by exogenous antigenic stimulation.

INTRODUCTION

In every animal, even in virtually "antigen-free" mice such as germ-free mice fed a chemically defined ultrafiltered diet, "background" immunoglobulin (Ig)-secreting cells occur in the various lymphoid organs (1). These background Ig-secreting cells account for the circulating and secretory Ig (2).

To study the influence of exogenous antigenic stimulation on the number and specificities of the background Ig-secreting cells, we have compared three groups of BALB/c mice that were kept under different conditions with regard to the extent of exogenous antigenic stimulation. These groups consisted of (a) germ-free mice fed a chemically defined ultrafiltered diet (GF-CD), (b) specific pathogen-free mice fed an autoclaved natural ingredient diet (SPF-NI), and (c) mice kept under nonbarrier con-

ventional breeding conditions and fed a nonautoclaved natural ingredient diet (CV-NI).

Earlier studies with such mice have shown that the number of background IgM-secreting cells as determined in the protein A–plaque assay is relatively constant and independent of the exogenous antigenic load (3, 4). However, the numbers of background IgG- and IgA-secreting cells appeared to be directly related to the exogenous antigenic load (3, 4).

Studies employing plaque assays specific for differently haptenized sheep red blood cells (SRBC) showed that the frequencies of antigen-specific IgM-secreting cells were virtually the same in GF-CD and CV-NI mice (3, 4). However, there were some inherent technical limitations in these studies. First, in naive mice only the number of IgM-secreting cells can be determined reliably with antigen-specific plaque assays. Since the number of IgM-secreting cells is relatively large when compared to the numbers of IgG- and IgA-secreting cells, indirectly developed background antigen-specific plaques (IgG, IgA) usually do not significantly exceed the number of directly developed (IgM) background antigen-specific plaques. The greatest numerical differences between the GF-CD mice and the control groups, however, were found for the IgG- and IgA-secreting cells. Second, in plaque assays the antigen has to be coupled efficiently to the target SRBC, which is not always possible. Therefore, usually only small haptens are used.

The ELISA–plaque assay overcomes these difficulties. In this assay the detection of Ig-secreting cells of all isotypes is completely dependent on the use of developing antibodies. This allows the detection of IgG and IgA antibody-secreting cells without the simultaneous development of ELISA plaques by IgM antibody-secreting cells. Therefore we employed this assay to extend our previous studies on the specificity repertoire of background Ig-secreting cells. The frequencies of antigen-specific IgM-, IgG-, and IgA-secreting cells were determined not only for the antigen DNP27-BSA, but also for the two idiotypes determined by the monoclonal anti-idiotypic antibodies Ac38 and Ac146.

MATERIALS AND METHODS

Mice. BALB/c AnN mice, which were originally obtained from the University of Wisconsin (Madison, WI), were reared and maintained germ-free in the Lobund Laboratory, University of Notre Dame (Notre Dame, IN) and fed a chemically defined ultrafiltered "antigen-free" diet consisting of L489-E14Se and LADEK 69E6 (GF-CD) as described in detail (5), or were specific pathogen free and fed the autoclaved natural ingredient L-485 (6) (SPF-NI). Other SPF BALB/c mice were obtained from Bomholtgard, Ltd. (Ry, Denmark) and were reared and maintained under clean, conventional, stable conditions and fed a conventional nonautoclaved diet AMII (CV-NI, Hope Farm, Woerden, The Netherlands) at the Department of Cell Biology, Immunology and Genetics, Erasmus University (Rotterdam, The Netherlands). (C57BL/Rij × CBA/Rij) F_1 mice were purchased from the Radiobiological Institute TNO (Rijswijk, The Netherlands) and kept under conventional conditions. Both male and female mice were used between 8 and 12 weeks of age.

Preparation of lymphoid cell suspensions. Cell suspensions of spleen, femoral and tibial bone marrow (BM), mesenteric lymph nodes (MLN), and peripheral lymph nodes (PLN) were prepared as previously described (7). The total number of nucle-

ated cells was counted with a Model BZI Coulter counter (Coulter Electronics, Harpenden, Berks, England).

ELISA–plaque assay. The ELISA–plaque assay was performed as described by Sedgwick and Holt (8) with some modifications. The reverse ELISA–plaque assay (9) for enumeration of the total number of Ig-secreting cells and the antigen-specific ELISA–plaque assay were performed in essentially the same way. Briefly, PVC microtiter plates (Type III, Flow Laboratories, Amstelstad, Zwanenburg, The Netherlands) were incubated with saturating amounts of coating ligand (see Antigens and antisera) in phosphate-buffered saline (PBS) for 1 hr at 37°C. After washing with PBS, the plates were incubated with PBS containing 1% of bovine serum albumin (PBS-BSA, Organon, Oss, The Netherlands) for 30 min at 37°C to block remaining binding sites. After washing with PBS, lymphoid cells diluted in RPMI 1640 supplemented with 1% BSA were added to the wells and incubated for 2–4 hr at 37°C in a vibration-free, 5% CO_2 incubator. The maximum number of nucleated cells per well was 5 million, because this was the highest number for which we found a good correlation between the number of cells plated per well and the number of spots per well. When more cells had to be tested, more wells were used and the results from the replicate wells were combined. After incubation the cells were washed away with H_2O containing 0.05% Tween 20 and subsequently the plates were washed three times with PBS–BSA with 0.05% Tween (PBS–BSA–Tw).

Hereafter the wells were incubated with isotype-specific goat anti-mouse Ig antisera (see Antigens and antisera), conjugated to alkaline phosphatase (AP, appropriately diluted in PBS–BSA–Tw) either for 15–18 hr at room temperature or for 2 hr at 37°C. After washing with PBS–BSA–Tw the wells were filled with a substrate–agarose mixture that consisted of 2.3 m*M* 5-BCIP (No. B 8503, Sigma, St. Louis, MO), 0.1 *M* amino-2-methyl-1-propanol (AMP), 0.01 m*M* $MgCl_2$, 0.01% Triton X-405, and 0.6% agarose (No. A 6013, Sigma). After 1 hr of incubation at 37°C clear blue spots were visible. The spots were counted using a reverse microscope or a colony viewer (Bellco Glass, Vineland, NJ).

Antigens and antisera. For enumerating the total number of Ig-secreting cells of a certain class in the reverse ELISA–plaque assay (9) a monoclonal rat anti-mouse κ light chain (226) (10) was used as the coating ligand. This antibody had been purified by affinity chromatography.

Dinitrophenyl (DNP) was coupled to BSA in a molar ratio of 27:1 (DNP27-BSA) according to the procedure of Eisen *et al.* (11).

To detect idiotype-positive Ig-secreting cells, the mouse monoclonal antibodies Ac146(IgG1, κ) and Ac38(IgG1, κ) (kindly provided by Dr. K. Rajewsky) that are both specific for the idiotype of B1-8 (IgM, λ, anti-NP) (12) were purified by affinity chromatography on a Protein A–Sepharose column. These two monoclonals recognize different but overlapping idiotopes of B1-8. All coating ligands were used at 10 μg/ml and could be reused three times without significant loss of signal.

The isotype-specific goat anti-mouse Ig AP antisera used (Southern Biotechnology Associates, Ltd., Birmingham, AL) were all tested for their specificity for the different Ig (sub)classes with appropriate myeloma or hybridoma cells in ELISA–plaque assays.

Protein A–plaque assay. The protein A–plaque assay was performed as described by Van Oudenaren *et al.* (13). Rabbit anti-mouse antisera specific for the different Ig

TABLE 1

Comparison of the Sensitivity of the Reverse ELISA–Plaque Assay (E-PFC) and the Protein A–Plaque Assay (PFC)

Isotype	E-PFC/spleen ($\times 10^{-3}$)	PFC/spleen ($\times 10^{-3}$)
IgM	588 ± 57^a	552 ± 43
IgG	149 ± 23	107 ± 6
IgA	471 ± 42	426 ± 43

[a] Figures represent the arithmetic means (±SEM) of five individual samples of the pooled spleens of five 12-week-old (C57BL/Rij × CBA/Rij)F_1 mice.

isotypes were tested for their specificity with the appropriate myeloma or hybridoma cells in the protein A–plaque assay.

Calculation of the total number of Ig-secreting cells and frequency determination of the antigen-specific or idiotype-positive Ig-secreting cells. The total number of Ig-secreting cells of a given class per organ was calculated by using the number of Ig-secreting cells in the reverse ELISA–plaque assay, the number of cells tested in the ELISA–plaque assay, and the total cell yield per organ. Ig-secreting cells in the BM were determined in the femoral and tibial BM. To calculate the number of Ig-secreting cells in the total BM from the number of Ig-secreting cells found in both femurs and tibiae we used a conversion factor of 5.4 (7).

The frequencies of antigen-specific and idiotype-positive Ig-secreting cells of a given class were determined in the appropriate ELISA–plaque assays and calculated as the ratio of the specific Ig-secreting cells to the total number of Ig-secreting cells of that particular Ig class.

Statistical analysis. The differences among the experimental groups of mice were evaluated with the Student *t* test.

RESULTS

Comparison of the Sensitivity of the Protein A– and ELISA–Plaque Assays

In order to compare the results obtained with the ELISA–plaque assay with earlier results obtained with the protein A–plaque assay, we compared the sensitivity of both assays directly. The results show that we detected at least equal numbers of background IgM- and IgA-secreting cells and even some more IgG-secreting cells with the ELISA–plaque assay than with the protein A–plaque assay (Table 1).

Background Ig-Secreting Cells in Various Lymphoid Organs

By means of the reverse ELISA–plaque assay the total numbers of "background" IgM-, IgG-, and IgA-secreting cells in the spleen, BM, MLN, and PLN of 8- to 12-week-old GF-CD, SPF-NI, and CV-NI BALB/c mice were determined. In the spleen we also determined the number of cells secreting the different subclasses of IgG. The results are shown in Table 2. Although in all organs tested, except for the PLN, the numbers of IgM-secreting cells were not significantly different among the experimental groups of mice, the numbers of (subclasses of) IgG- and IgA-secreting cells were reduced in the mice with a reduced exogenous antigenic load. There was a strict corre-

TABLE 2

Numbers of "Background" Ig-Secreting Cells in Spleen, BM, MLN, and PLN of Germ-Free BALB/c Mice Fed a Chemically Defined Ultrafiltered Diet (GF-CD), SPF BALB/c Mice Fed an Autoclaved Natural Ingredient Diet (SPF-NI), and Conventional BALB/c Mice Fed a Nonautoclaved Natural Ingredient Diet (CV-NI)

		E-PFC $\times 10^{-3}$/organ			Ratio	Ratio
Organ	Isotype	GF-CD	SPF-NI	CV-NI	SPF-NI/GF-CD	CV-NI/GF-CD
Spleen	IgM	267 ± 50[a]	266 ± 43	469 ± 75	1 ($P > 0.1$)[b]	1.8 ($P > 0.1$)
	IgG1	0.1 ± 0.1	1.7 ± 0.2	25 ± 5	17 ($P < 0.005$)	250 ($P < 0.05$)
	IgG2a	0.2 ± 0.08	2.1 ± 0.4	29 ± 6	11 ($P < 0.01$)	145 ($P < 0.05$)
	IgG2b	0.2 ± 0.09	3.1 ± 1.0	20 ± 5	16 ($P < 0.05$)	100 ($P < 0.05$)
	IgG3	0.1 ± 0.07	3.2 ± 0.5	14 ± 3	32 ($P < 0.005$)	140 ($P < 0.05$)
	IgA	0.3 ± 0.3	89 ± 26	240 ± 41	297 ($P < 0.05$)	800 ($P < 0.01$)
BM	IgM	97 ± 25	84 ± 24	61 ± 6	0.87 ($P > 0.1$)	0.63 ($P > 0.5$)
	IgG	2.0 ± 0.4	37 ± 13	73 ± 11	19 ($P < 0.05$)	37 ($P < 0.005$)
	IgA	1.5 ± 0.7	79 ± 24	107 ± 38	53 ($P < 0.05$)	71 ($P < 0.05$)
MLN	IgM	4.6 ± 2.5	8.2 ± 6.6	4 ± 1.5	2 ($P > 0.1$)	0.87 ($P > 0.1$)
	IgG	0.2 ± 0.1	4.9 ± 1.6	58 ± 18	25 ($P < 0.05$)	290 ($P < 0.05$)
	IgA	0.3 ± 0.1	3.9 ± 1.6	31 ± 2	13 ($P < 0.05$)	103 ($P < 0.001$)
PLN	IgM	0.7 ± 0.2	4 ± 0.6	N.D.	6 ($P < 0.01$)	—
	IgG	0.04 ± 0.08	2.8 ± 0.9	N.D.	40 ($P < 0.05$)	—
	IgA	0.01 ± 0.01	1.6 ± 0.05	N.D.	270 ($P < 0.001$)	—

[a] Numbers represent the arithmetic means (±SEM) of three to seven individual experiments. In each experiment the organs of six animals of each group were pooled.

[b] Statistical analysis was performed with the Student t test.

lation between the antigenic load of the mice tested and the number of IgG- and IgA-secreting cells detected.

Frequency of DNP27-BSA-Specific Ig-Secreting Cells

The frequencies of DNP27-BSA-specific Ig-secreting cells among the different isotypes were determined in the spleen and BM of GF-CD, SPF-NI, and CV-NI mice. The results are shown in Table 3.

In the spleen the frequencies of DNP27-BSA-specific IgM-secreting cells were not significantly different among the different groups of mice. For the IgG- and IgA-secreting cells, however, substantial differences were found among the three groups of mice. In the GF-CD mice the frequencies of DNP27-BSA-specific IgG-secreting cells were 37 and 1250 times higher than those in the SPF-NI and CV-NI controls, respectively. Although many times more IgG-secreting cells were tested, DNP27-BSA-specific IgG-secreting cells were hardly detectable in CV-NI mice. The frequencies of DNP27-BSA-specific IgA-secreting cells in the spleen of GF-CD mice were 37 and 23 times higher than those in SPF-NI and CV-NI mice, respectively.

In the BM the frequencies of DNP27-BSA-specific IgM-secreting cells were also comparable among the three groups of mice investigated. The frequency of DNP27-BSA-specific IgG-secreting cells was 16 times higher in GF-CD mice than in SPF-NI

TABLE 3

Frequency of DNP27-BSA-Specific Ig-Secreting Cells in the Spleen and BM of Germ-Free BALB/c Mice Fed a Chemically Defined Ultrafiltered Diet (GF-CD), SPF BALB/c Mice Fed an Autoclaved Natural Ingredient Diet (SPF-NI), and Conventional BALB/c Mice Fed a Nonautoclaved Natural Ingredient Diet (CV-NI)

	Ig-secreting cells	GF-CD		SPF-NI		CV-NI	
		Cells tested[a]	Frequency	Cells tested	Frequency	Cells tested	Frequency
Spleen	IgM	222	1 in 143 (±32)[b]	243	1 in 137 (±28)	52	1 in 65 (±22)
	IgG	1.9	1 in 48 (±18)	27	1 in 1766 (±417)[c]	989	1 in 60000 (±22060)[d]
	IgA	1.5	1 in 93 (±37)	100	1 in 3433 (±2355)[c]	465	1 in 2118 (±327)[e]
BM	IgM	8.9	1 in 30 (±1)	6.3	1 in 21 (±6)	4.2	1 in 104 (±44)
	IgG	1.6	1 in 71 (±53)[c]	5.8	1 in 1112 (±706)[c]	28	N.D.[f]
	IgA	1.1	1 in 113[g]	12	1 in 228 (±53)[c]	6.4	1 in 1940 (±1660)

[a] Total number of Ig-secreting cells ($\times 10^{-3}$) of a particular isotype evaluated for DNP27-BSA-specific Ig-secreting cells.

[b] Unless indicated otherwise, figures represent the arithmetic means (±SEM) of three to seven individual experiments. In each experiment the organs of six animals of each group were pooled.

[c] Figures represent the arithmetic means (±SEM) of two individual experiments. In one other experiment DNP27-BSA-specific Ig-secreting cells were not detectable among the tested Ig-secreting cells of this isotype.

[d] Figures represent the arithmetic means (±SEM) of four individual experiments. In three other experiments DNP27-BSA-specific IgG-secreting cells were not detectable in the samples tested.

[e] Figure represents the arithmetic mean (±SEM) of five individual experiments. In two other experiments DNP27-BSA-specific IgA-secreting cells were not detected in the samples tested.

[f] DNP27-BSA-specific IgG-secreting cells were not detected in the samples tested.

[g] Figure represents the frequency detected in one experiment. In two other experiments DNP27-BSA-specific IgA-secreting cells were not detected.

mice. In the BM of the CV-NI mice we could not detect any DNP27-BSA-specific IgG-secreting cells, although a total of 28,000 IgG-secreting cells were tested in seven individual experiments. The frequency of DNP27-BSA-specific IgA-secreting cells in the BM was comparable between GF-CD and SPF-NI mice, while in the CV-NI mice a substantially lower frequency was found.

Frequency of Idiotype-Positive Ig-Secreting Cells

With the use of the monoclonal antibodies Ac146 and Ac38, which recognize different but overlapping idiotopes of the monoclonal antibody B1-8 (anti-NP), the frequencies of these idiotypes among the IgM- and IgA-secreting cells in the spleen of GF-CD, SPF-NI, and CV-NI mice were determined (Table 4). The frequency of idiotype-positive IgG-secreting cells could not be determined, as the anti-idiotypic antibodies themselves belong to the IgG1 subclass.

The frequency of Ac146- and Ac38-positive IgM-secreting cells did not differ significantly ($P > 0.05$) among the three groups of mice. The frequency of Ac146-positive IgA-secreting cells in the spleen of GF-CD mice, however, was 139 times higher than that in both control groups of mice, while the frequencies of Ac38-positive IgA-

TABLE 4

Frequency of B1-8 Idiotype-Positive IgM- and IgA-Secreting Cells in the Spleen of Germ-Free BALB/c Mice Fed a Chemically Defined Ultrafiltered Diet (GF-CD), SPF BALB/c Mice Fed an Autoclaved Natural Ingredient Diet (SPF-NI), and Conventional BALB/c Mice Fed a Nonautoclaved Natural Ingredient Diet (CV-NI)

Anti-idiotype	Ig-secreting cells	GF-CD	SPF-NI	CV-NI
Ac146	IgM	1 in 992 (±815)[a]	1 in 3151 (±2195)	1 in 5072 (±2227)
	IgA	1 in 99 (±65)	1 in 13734 (±10866)	1 in 13849 (±2233)[b]
Ac38	IgM	1 in 1287 (±900)	1 in 3138 (±2258)	1 in 4179 (±1327)
	IgA	1 in 169 (±157)	1 in 8519 (±5539)	1 in 86640 (±81361)

[a] Figures represent the arithmetic means (±SEM) of the data obtained in three or four separate experiments. In each experiment the spleens of six mice of each group were pooled.

[b] Significantly different ($P < 0.05$) between CV-NI and GF-CD mice.

secreting cells were 50 and 513 times higher in the spleen of the GF-CD mice than those in the spleen of the SPF-NI and CV-NI mice, respectively.

DISCUSSION

The aim of this study was to further extend our previous studies on the role that exogenous antigens might play in the specificity repertoire of "background" Ig-secreting cells. With the use of the ELISA–plaque assay we were able to study the specificity repertoire of Ig-secreting cells with a specificity that is weakly expressed in the repertoire, i.e., specificity for a particular anti-idiotype.

With the use of the reverse ELISA–plaque assay no significant differences were found in the number of background IgM-secreting cells in the spleen, BM, and MLN when comparing 8- to 12-week-old GF-CD, SPF-NI, and CV-NI mice. However, the numbers of background IgG- and IgA-secreting cells in these organs were strongly reduced in the GF-CD mice as compared to both groups of control mice. SPF-NI mice were found to have lower numbers of IgG- and IgA-secreting cells than CV-NI mice. Thus there seems to be a strict correlation between the extent of exogenous antigenic load and the number of IgG- and IgA-secreting cells. These data confirm in general earlier results of our group (1, 4) and others (14) obtained with the protein A–plaque assay.

A clear discrepancy with previous data is the much more drastic reduction of the number of IgG-secreting cells in the spleen as compared with data obtained with the protein A–plaque assay. Pereira *et al.* (14) detected a similar reduction in the number of IgG- and IgA-secreting cells in the spleen of the GF-CD mice with the protein A–plaque assay. This discrepancy could be due to the batch of anti-IgG antiserum that we have previously used in the protein A–plaque assay. Although thoroughly tested for cross-reactivity with IgM, it might be that this antiserum still detected some IgM. Due to the preponderance of IgM-secreting cells in the spleen of GF-CD mice, a weak cross-reactivity might account for the development of a significant number of plaques by IgM-secreting cells using the putative anti-IgG antiserum. However, when tested with myeloma and hybridoma cells, no such cross-reactivity had been observed. The

generally lower production rate of Ig by *in vitro* cultured myeloma and hybridoma cells might account for this. This might have led to an overestimation of the number of IgG-secreting cells in the protein A–plaque assay. In the current experiments and in the experiments of Pereira *et al.* (14) subclass-specific antisera were used which might display less cross-reactivity.

Another reason for the decreased number of IgG-secreting cells found in the present study might be that we used a monoclonal anti-κ light chain antibody as catching antibody, under the assumption that in the mouse 95% of the Ig-secreting cells produce κ light chains. Table 1 shows that this seems to be a correct assumption. However, it might be that in GF-CD mice the κ/λ ratio among the IgG-secreting cells is smaller than that in the control mice.

In previous studies on background antigen-specific Ig-secreting cells the specificity repertoire was determined by plaque assays (1, 3). A drawback of this assay, however, is that low numbers of antibody-forming cells can be determined reliably for IgM only. This is due to the fact that simultaneous with the development of plaques by IgG and IgA antibody-secreting cells, plaques by IgM antibody-secreting cells develop in the plaque assay. Thus the figures obtained have to be corrected for the often relatively high number of IgM antibody-secreting cells.

In this paper the ELISA–plaque assay was used. In this assay the detection of Ig-secreting cells of all isotypes is completely dependent on the use of developing antibodies. This allows the detection of IgG and IgA antibody-secreting cells without the simultaneous development of ELISA–plaques by IgM antibody-secreting cells. We made use of this assay to determine frequencies of DNP27-BSA-specific Ig-secreting cells of all isotypes in the spleen and BM of GF-CD, SPF-NI, and CV-NI mice.

The results show that stimulation of the immune system by environmental antigens greatly influences the frequency of DNP27-BSA-specific IgG- and IgA-secreting cells, whereas the frequency of IgM anti-DNP27-BSA-secreting cells is not influenced. In GF-CD mice the frequency of anti-DNP27-BSA IgG- and IgA-secreting cells resembles that of the IgM-secreting cells, although the total numbers of IgG- and IgA-secreting cells were very much reduced. This suggests an at-random low rate of switching of DNP27-BSA-specific cells from IgM secretion to the secretion of other isotypes in these mice.

The relatively low frequency of DNP27-BSA-specific IgG- and IgA-secreting cells in SPF-NI and CV-NI mice indicates that environmental antigens preferentially stimulate Ig class switching of cells with specificities other than those that recognize DNP27-BSA.

The great differences that we found among the frequencies of DNP27-BSA-specific IgM-secreting cells (1 in 65), IgG-secreting cells (1 in 60,000), and IgA-secreting cells (1 in 2118) in CV-NI mice cannot be caused by differences in detection levels of the different assays. First, the frequencies of DNP27-BSA-specific IgM-, IgG-, and IgA-secreting cells in GF-CD mice were equally high. Second, we tested the sensitivity of the assays directly by comparing DNP27-BSA-specific hybridomas secreting IgM and IgG and they showed a comparable sensitivity (data not shown).

Furthermore, a difference was found between the frequencies of DNP27-BSA IgM-secreting cells in the spleen and bone marrow of GF-CD and SPF-NI mice. Other studies also showed differences between the specificity repertoires of the background IgM-secreting cells in the spleen and bone marrow (15). The underlying cause of the frequently observed difference in the specificity repertoires in spleen and bone marrow is unclear.

Studies with 1- to 2-year-old GF-CD mice, which have been shown to have more background IgG- and IgA-secreting cells than young GF-CD mice (1), show that the frequencies of DNP27-BSA-specific IgG- and IgA-secreting cells decrease while the total numbers of IgG- and IgA-secreting cells increase (data not shown). A possible increase in the endogenous antigenic stimulation during aging as reflected by a higher number of autoantibodies in aging mice (16) might influence the number and the specificity repertoire of background IgG- and IgA-secreting cells.

In the studies on the frequency of B1-8 idiotype-specific Ig-secreting cells it was shown that among the IgM-secreting cells no significant differences were found for the different groups of mice, although the frequencies tend to be lower with higher antigenic loads. The frequency of idiotype-positive IgA-secreting cells was substantially higher in GF-CD mice than in SPF-NI and CV-NI mice. A remarkable finding in these idiotype frequency studies was the greater variability for the frequencies of idiotype-positive Ig-secreting cells when compared to the DNP27-BSA determinations. This might be caused by the fact that the anti-idiotypic antibodies probably recognize only a single epitope. Consequently, individual mice will display a variability for such specificities greater than that of DNP27-BSA, which can be recognized by several different antibodies.

We realize that we have tested only a small number of antigens, but the striking differences between the frequencies of DNP27-BSA-specific IgM-, IgG-, and IgA-secreting cells in conventional mice, which we did not find in GF-CD mice, indicate that exogenous antigenic stimulation has a great influence on the specificity repertoire of the IgG- and IgA-secreting cells, but not on that of the IgM-secreting cells.

Recently it was suggested that the actual B-cell repertoire might be divided into two compartments (17). Our experiments indeed suggest two different subpopulations of background Ig-secreting cells.

The first compartment consists of B cells that are driven to develop into clones of Ig-secreting cells by endogenous stimuli. They consist mainly of IgM-secreting cells, although the low numbers of IgG- and IgA-secreting cells in GF-CD mice may also belong to this compartment. The endogenous stimuli may be provided by idiotypes, e.g., from maternal Ig transferred via the placenta or milk (18) or other self-antigens during the development of the immune system (19). Idiotypic interactions with T cells might also represent a driving force and/or play a regulating role (20). This autonomous compartment remains very stable during the life time and is relatively independent of exogenous antigenic stimuli. It can be speculated that the B cells involved belong to the naturally activated, autoreactive B cells as described by Portnoï (21).

The second compartment consists mainly of IgG- and IgA-secreting cells. The specificity repertoire of these cells most probably reflects the stimulation of the individuals' immune system by exogenous antigens. The relationship between the first and the second compartment remains to be established.

The specificity repertoire of the background IgG- and IgA-secreting cells in conventional mice is of great interest because it reflects the influence of exogenous antigenic stimulation on the immune system. Gnotobiotic animals, on the other hand, are very valuable for studying the development and regulation of the background IgG and IgA specificity repertoires under conditions of defined exogenous stimulation.

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CHAPTER 5

SERUM IMMUNOGLOBULIN LEVELS AND NATURALLY OCCURRING ANTIBODIES AGAINST CARBOHYDRATE ANTIGENS IN GERM-FREE BALB/c MICE FED CHEMICALLY DEFINED ULTRAFILTERED DIET

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ABSTRACT

This paper investigates the influence of exogenous antigenic stimulation on the serum immunoglobulin (Ig) levels and the levels of circulating natural antibodies against carbohydrate antigens. Therefore we made use of BALB/c mice raised in a germfree environment and fed a chemically defined, ultrafiltered diet (GF-CD). These mice had normal serum IgM levels, but IgG and IgA levels were approximately 5% of conventionally reared littermates. The concentration of all four IgG isotypes were equally low. The variable part of the heavy chains of naturally occurring BALB/c antibodies against a number of carbohydrate antigens, including 3-fucosyllactosamine (3-FL), levan and dextran are encoded by V_H441 and these antibodies express cross reactive idiotopes recognized by the monoclonal antibodies 6C4 and 6B1. Antibodies against levan and dextran were lower in GF-CD than in conventional mice, but levels of anti-3FL antibodies, and 6C4 and 6B1 idiotopes, were comparable to those in conventional animals. Peptidoglycan polysaccharide complexes (PPC) are carbohydrate antigens of bacterial origin, like levan and galactan. Naturally occurring antibodies against PPC were found in the serum of conventional mice, but were severely reduced in GF-CD mice. The results indicate that most naturally occurring antibodies against carbohydrate antigens of bacterial origin found in conventional mice are caused by exogenous stimulation.

1. INTRODUCTION

Antibodies against carbohydrate antigens occur naturally in mammalian sera, and are thought to result from stimulation by environmental antigens, particularly by microbial organisms that colonize the gastrointestinal tract. Levels of natural antibodies that bind to human blood group B antigen were low in chickens raised in a germfree environment, and rose after the chickens were fed E. coli that contained a B-like antigen (1). Antibodies against a soluble bacterial carbohydrate fraction can be found in human serum (2). Antibodies against a number of carbohydrate determinants, including levan, dextran, galactan and 3-fucosyllactosamine (3-FL) are found naturally in the sera of unimmunized mice (3,4). The V_H regions of many of these antibodies are encoded by the V_H gene V_H441 (5,6,7), and the antibodies express crossreactive idiotopes that are associated with their heavy chains (8). The expression of crossreactive idiotopes on these anti-carbohydrate antibodies suggested that they might participate in an idiotypic network.

Stimulation of mice by exogenous (bacterial) antigens can be excluded by raising them germfree and feeding them a chemically defined ultrafiltered diet (GF-CD). BALB/c mice can be kept under these conditions for more than ten generations (9). We reported previously that equal numbers of IgM-secreting cells were found in GF-CD and conventional mice, but that the numbers of IgG- and IgA-secreting cells are severely reduced in GF-CD mice compared to conventional animals (10). Also the frequency of antigen-specific IgM-secreting cells specific for differently haptenated sheep red blood cells and DNP27-BSA were comparable between GF-CD and conventional mice, but great differences were found for the frequencies of DNP27-BSA-specific IgG- and IgA-secreting cells between these groups of mice (11).

In this study we examined the serum levels of Ig in GF-CD mice, and analyzed the effects of the 'antigen-free' environment on the appearance of naturally occurring antibodies against several carbohydrate antigens.

Total serum Ig levels in unimmunized mice were determined previously by relatively insensitive methods like rocket electrophoresis (12) and Mancini (13). Van Snick and Masson used a radio immunoassay to determine Ig levels in germfree and conventional mice from different origins and showed an influence of the exogenous antigenic environment on the concentration of IgG and IgA (14). Because our GF-CD mice were expected to have even lower levels of IgG and IgA, we developed a sensitive sandwich ELISA to determine total serum levels. The GF-CD animals had normal levels of IgM, but their IgG and IgA levels were depressed to 5% of that of conventional mice. IgM-antibodies against 3-FL were found in a comparable amount in conventional and GF-CD mice, although antibodies against levan, galactan and PPC

were depressed in the latter.

2. MATERIALS AND METHODS

2.1. Mice

BALB/cAnN mice, that were obtained from the University of Wisconsin and had been obtained originally from the National Institutes of Health in 1983, were reared and maintained in the Lobund Laboratory, University of Notre Dame (Notre Dame, IN) either germfree and fed chemically defined ultrafiltered "antigen-free" diet L489-E14Se and LADEK 69E6 (GF-CD) as described in detail (9), or specific pathogen-free (SPF) and fed natural ingredient L-485.

2.2. Measurement of serum immunoglobulin levels

Serum Ig levels were determined in a sandwich ELISA. In this ELISA monoclonal rat anti-mouse-kappa (226; 10 μg/ml in PBS) was coated to PVC microtiterplates (Flow Laboratories, Irvine, Scotland), and the plates were blocked with PBS containing 1% BSA. Plates were then incubated with serial dilutions of mouse serum or affinity purified monoclonal antibodies and, after washing, incubated with goat-anti-mouse Ig-isotype-specific antisera coupled to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). Plates were developed with the Sigma 104 phosphatase substrate. OD405 was read in a MCC multiscan (Flow Laboratories). All incubations were for one hour at 37°C, except for the blocking step where the incubation time was 30 minutes.

Absolute concentrations were determined by comparison with standard concentrations of affinity purified monoclonal antibodies of the different isotypes. As positive controls the following cell lines were used: Sp603 (IgM,κ); Ac38 (IgG1,κ); P1.17 (IgG2a,κ; ATCC No. TIB-10); MPC11 (IgG2b;κ ; ATCC No. CCL167); AFLA IV 307 (IgG3,κ); 11D1 (IgA,κ).

2.3. Anti-carbohydrate antibodies

The ELISA assays for measurement of antibodies against levan, galactan and 3-FL, and for measurement of idiotope levels in serum, have been described in detail (15).

Isolation of peptidoglycan-polysaccharide complexes (PPC) from human faecal flora has been described in detail (2). Antibodies against PPC were determined in ELISA. PPC was coated to polystyrene 96 wells plates by drying overnight at 37°C. Sera, serially diluted in PBS, 1% Tween-20, were incubated for one hour at 37°C and, after washing, goat-anti-mouse Ig coupled to peroxidase was added. Plates were developed with 0-phenylene-diamin substrate and measured at OD 492 nm.

3. RESULTS

3.1. Levels of serum immunoglobulins

For the measurement of the serum Ig levels we have developed a sensitive sandwich ELISA. The specificity and sensitivity for the different isotypes are shown in Figure 1. The concentration of serum Ig was determined in 8-12 wk old GF-CD and CV-NI BALB/c mice (Table 1). The IgM concentration did not differ significantly between GF-CD and CV-NI BALB/c mice. In contrast, all subclasses of IgG and IgA were severely reduced in GF-CD mice compared to their conventional age-matched controls. Furthermore, some 15-months-old mice, that were used for breeding purposes and some pregnant GF-CD and CV-NI mice that were available in our breeding colony were also tested. In old GF-CD and CV-NI mice the IgM concentration

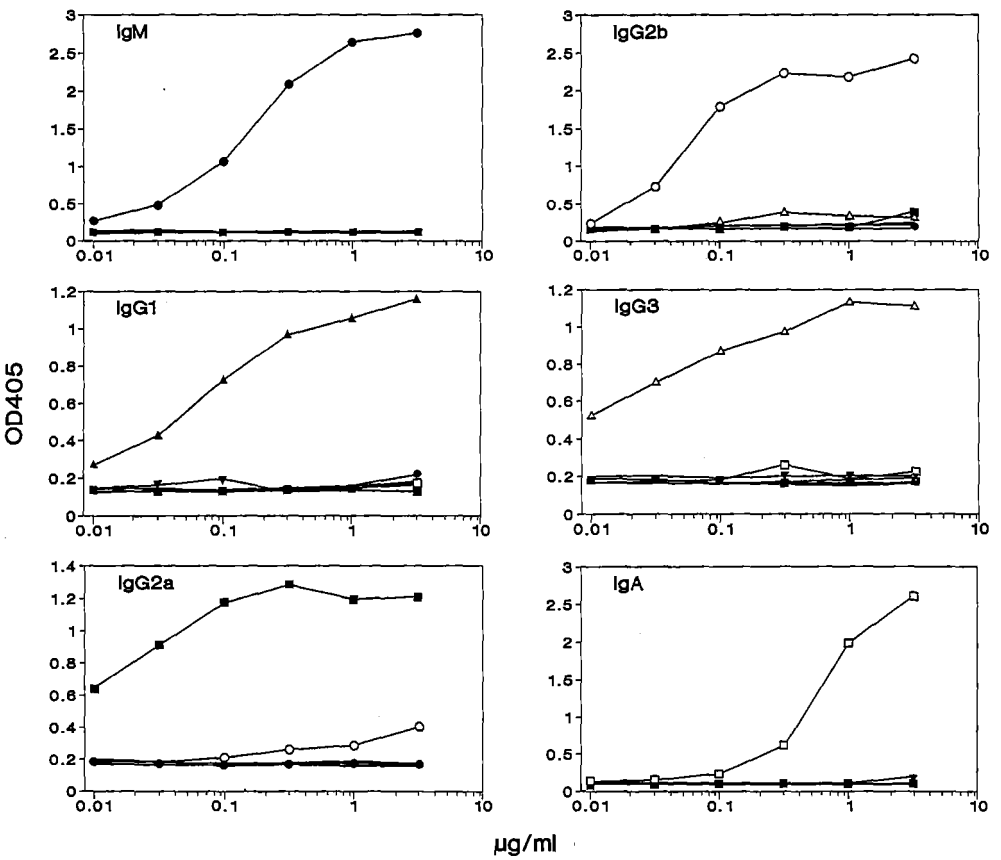

Figure 1

Specificity and sensitivity of the sandwich ELISA for mouse isotypes. Positive controls for IgM (●), IgG1 (▲), IgG2a (■), IgG2b (○), IgG3 (△), IgA (□) and PBS (▼; negative control) were tested in all isotype-specific ELISA assays.

was higher than in young mice (1123 ± 105 and 564 ± 173 μg/ml, respectively). The IgG and IgA levels in old GF-CD mice were not significantly different from young GF-CD mice, while old CV-NI mice showed a threefold increase in the IgG and IgA levels (data not shown). In pregnant GF-CD mice, an increase of the IgG1 and IgG2a concentrations were found (598 ± 69 and 117 ± 5 μg/ml, respectively).

TABLE 1

Serum immunoglobulin levels in germfree BALB/c mice fed chemically defined ultrafiltered diet (GF-CD) and conventional mice (CV-NI)

Isotype	GF-CD	CV-NI
IgM	323 ± 32[a]	284 ± 35
IgG1	38 ± 7	441 ± 44
IgG2a	12 ± 1	384 ± 109
IgG2b	2 ± 0.3	111 ± 36
IgG3	4 ± 1	101 ± 21
IgA	3 ± 0.5	40 ± 5

a. Serum Ig concentration expressed as μg/ml ± SE of 6 GF-CD and 7 CV-NI 8-12 wk old mice.

3.2. Anti-carbohydrate antibodies and idiotypes

A summary of the data on antibody and idiotope levels is presented in Figure 2, and titrations of sera from individual mice are presented in Figure 3. BALB/c mice raised under conventional conditions had naturally occurring antibodies against 3-FL, levan and galactan, as noted previously (4). The mean levels of antibodies against levan and galactan in the GF-CD mice were approximately 20 and 33%, respectively, of those in conventional mice. Anti-3-FL antibodies in GF-CD mice were comparable to those of conventional mice (Figures 2A, 3A and 3B) and possibly higher. Similar data were obtained with sera from three other GF-CD and conventional mice (data not shown).

The anti-3-FL antibodies found in GF-CD mice were shown to be of the IgM class (data not shown). The cross-reactive idiotopes 6C4 and 6B1, which are associated with the V_H441 encoded Ig heavy chain, were also present in comparable quantities in both GF-CD and conventional mice (Figure 2B).

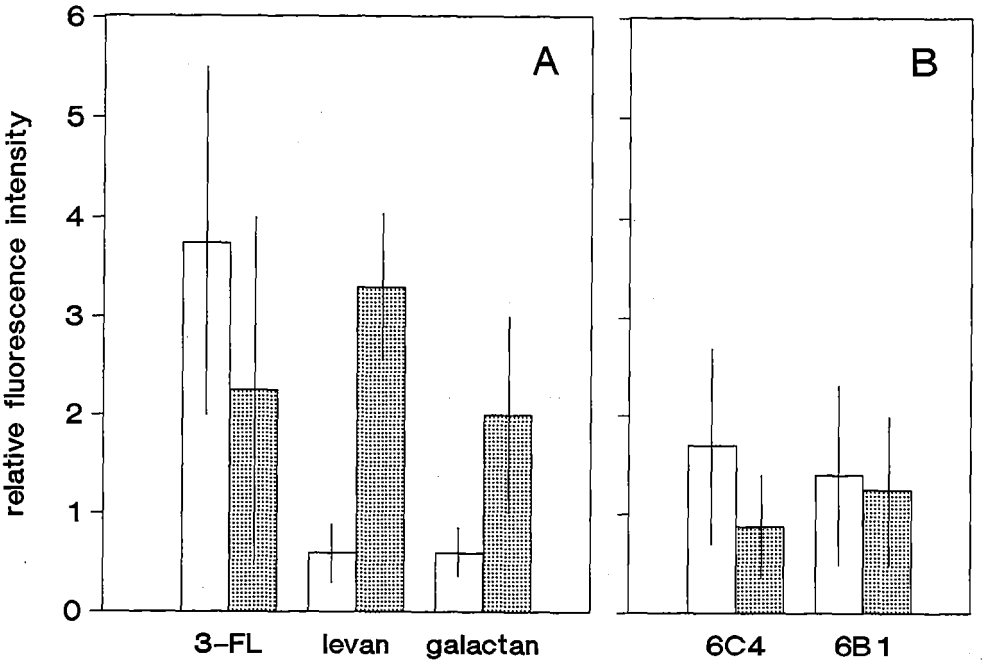

Figure 2

Levels of antibodies against carbohydrate antigens (A) and idiotopes (B) in the sera of GF-CD and conventional mice. Each bar represents the mean and standard error of assays on sera from six mice. Open columns represent GF-CD mice and dotted columns conventional mice.

3.3. Antibodies against peptidoglycan polysaccharide complexes

Antibody titrations against PPC in GF-CD and conventional mice are shown in Figure 4. Conventional mice had naturally occurring circulating antibodies against PPC, while in GF-CD mice no anti-PPC antibodies could be detected.

4. DISCUSSION

Serum Ig concentrations in normal, unimmunized mice have mostly been determined by relatively insensitive methods like rocket-electrophoresis (12), Ouchterlony (16) and radial immunodiffusion (13). In these studies an influence of T cells, the antigenic environment and the age of the mice has been shown. Van Snick and Masson, using an RIA technique, have shown that levels of IgG and IgA were lower in germfree DBA/2 mice than in conventional controls (14). In order to measure the

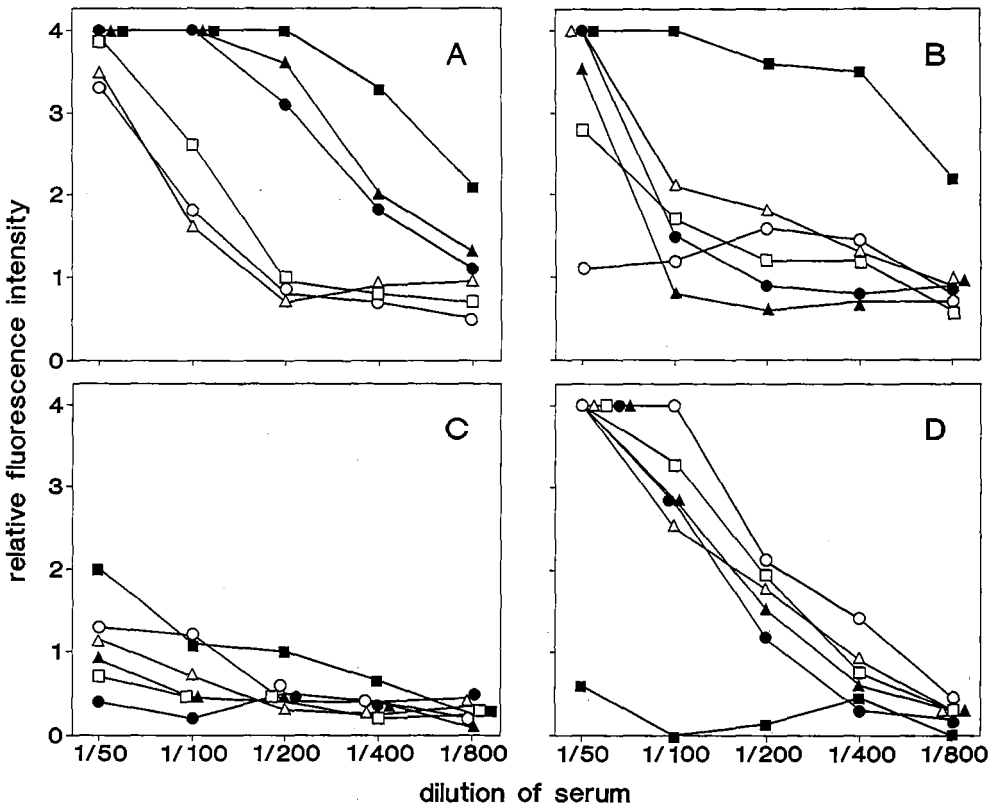

Figure 3

Titrations of individual sera against 3-FL (panels A and B) and levan (panels C and D). The sera in Figures A and C were obtained from GF-CD mice and those in panels B and D from conventional mice.

low levels of IgA and IgG-subclasses in the antigenically even more deprived GF-CD mice we have developed a sensitive sandwich ELISA.

The serum Ig concentrations of conventional mice that were obtained with our sandwich ELISA fall within the same range as determined by others, although there is great variation in the values obtained by different investigators (12-14,16). Environmental factors are shown to be important for the values obtained in conventional mice (14). Our conventional mice are directly derived from the GF-CD colony and kept under very clean SPF conditions, which might explain the relatively low concentrations of IgG we have found. The serum Ig levels in GF-CD mice are in agreement with the numbers of Ig-secreting cells of the different isotypes in the spleen

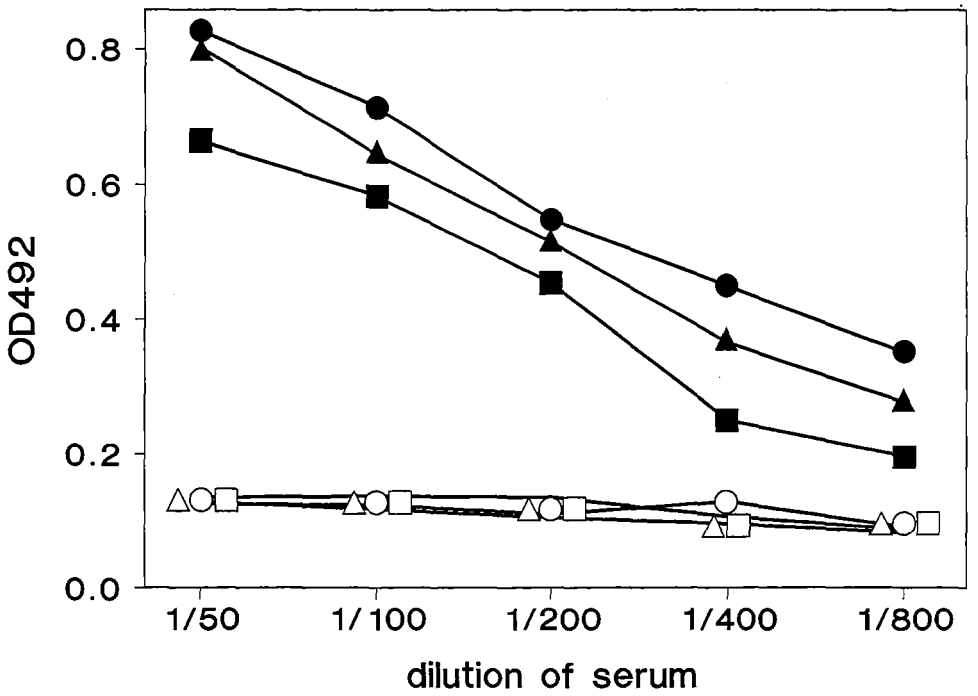

Figure 4

Titrations of sera from individual mice against PPC. Open symbols represent GF-CD mice and closed symbols conventional mice.

of these mice (11). In conventional mice, however, a clear discrepancy was found between the concentrations of the different Ig isotypes in the serum and the numbers of Ig-secreting cells of the different isotypes in the spleen. In the serum, IgG is the most prominent isotype, while IgM is the most abundant isotype among the splenic Ig-secreting cells. This difference can only partially be explained by the 2-3 times shorter halflives of serum IgM compared to IgG (17). The data suggests that in GF-CD mice the serum Ig concentration reflects the production of the splenic Ig-secreting cells, while in conventional mice other organs, such as bone marrow and lymph nodes contribute considerably to the serum concentration, especially for IgG and IgA (18).

Pregnant GF-CD and conventional mice have been shown to have an increase in the number of Ig-secreting cells (19). The serum Ig levels were found to be also elevated during pregnancy. In pregnant GF-CD mice IgG1 and IgG2a levels were higher than in virgin controls. This seems to be a reversible elevation because in old GF-CD mice which were used earlier for breeding purposes the concentration of IgG1 and IgG2a was not significantly different from young, virgin GF-CD mice.

Naturally occurring antibodies against carbohydrate antigens are thought to arise in response to stimulation by environmental antigens. Galactan is found in many plant and wood products (20), levan is produced by enteric bacteriae (4) and PPC is derived from the human anaerobic intestinal flora (2). It is therefore not surprising that antibodies against these determinants were hardly or not detectable in GF-CD mice. We are not aware of data concerning the occurrence of the 3-FL determinant in bacteria, but this antigen is present as autoantigen in the kidneys of BALB/c mice (21). The production of anti-3-FL antibodies may be stimulated by internal ligands that bind to surface Ig of B cells, autoantigen and/or anti-idiotypic antibodies. B cells producing polyreactive IgM antibodies reacting both with autoantigens and bacterial antigens can be isolated from newborn mice and from adult GF-CD mice after LPS stimulation (22). These B cells committed to the production of multireactive antibodies do not give rise to significant anti-bacterial antibody titers in the serum of GF-CD mice. Newborn serum has not been investigated for multireactivity or anti-bacterial activity. Whether the anti-3-FL IgM-antibodies found in the serum of GF-CD mice belong to this class of antibodies is unknown. These multireactive antibodies are thought to be produced by Ly-1^+ B lymphocytes (23). We have indeed observed a higher percentage of Ly-1^+ B cells in the spleen of GF-CD mice compared to conventional controls (6% vs 1%, unpublished observations). B cells that express complementary idiotopes are present early in ontogeny (reviewed in 24). In a previous paper it was suggested that anti-carbohydrate antibodies that display a common idiotope on their heavy chain may participate in a regulatory idiotypic network (3). The difference between levels of antibodies against 3-FL, levan and galactan indicates that an idiotypic network involving determinants shared by these antibodies is not the dominant factor regulating levels of these antibodies in GF-CD mice. It does not, however, rule out the existence of an idiotypic network involving determinants unique to the 3-FL antibodies, nor is it evidence against the operation of a network that recognizes shared determinants among these antibodies in conventional mice.

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CHAPTER 6

'BACKGROUND' Ig-SECRETING CELLS IN PREGNANT GERMFREE MICE FED A CHEMICALLY DEFINED ULTRAFILTERED DIET

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'Background' Ig-secreting cells in pregnant germfree mice fed a chemically defined ultrafiltered diet

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During syngeneic pregnancy the numbers of 'spontaneously' occurring ('background') Ig-secreting cells were determined in the spleen, bone marrow (BM) and mesenteric lymph nodes (MLN) of BALB/c mice that were kept under germfree conditions and fed a low molecular weight synthetic diet (GF-CD), SPF BALB/c mice fed autoclaved natural ingredient (SPF-NI) and conventional BALB/c mice fed natural ingredient (CV-NI). 'Background' Ig-secreting cells were enumerated in the protein A plaque assay and the specificity repertoire of the IgM-secreting cells was determined with plaque assays specific for differently haptenized sheep red blood cells (SRBC). The numbers of 'background' Ig-secreting cells, especially the IgG- and IgA-secreting cells, are very much reduced in the BM and MLN of GF-CD mice as compared to SPF-NI and CV-NI mice. During pregnancy the total number of Ig-secreting cells increased in all lymphoid organs tested, but the proportional increase was most prominent for the IgG- and IgA-secreting cells in the BM and MLN of the GF-CD mice. This increase could only be due to their pregnant state since all environmental antigenic influences are excluded in GF-CD mice. No changes were found in the background specificity repertoire of the IgM-secreting cells during pregnancy. This suggests a polyclonal activation of the Ig-secreting cells during pregnancy. The reason for this activation remains obscure, but it has to be endogenous. Pregnancy apparently induces a new steady state of the immune system, which can be most properly investigated in GF-CD mice.

Key words: *Ig-secreting cells, plaque assay, germ-free mouse, syngeneic pregnancy*

Introduction

The maternal lymphoid organs change markedly during pregnancy. The thymus gland decreases in size, while the spleen and several lymph nodes temporarily increase in size (Maroni and De Sousa, 1973). The immunological implications of these changes are still poorly understood. Modification of maternal immune reactivity does not seem to be due to an inability of the maternal lymphocytes to respond to antigenic stimulation (Gambel and Ferguson, 1982). Parous mice respond to

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embryonic and paternal antigens as shown for cell-mediated immune responses by cytotoxic effects on cultured embryonic cells (Hamilton and Hellström, 1977) as well as for humoral responses by alloantibodies to placental antigens (Gill and Repetti, 1979). Less is known about the B cells that produce these antibodies. McLean et al. (1980) showed a significant B cell proliferation during pregnancy, although Chatterjee-Hasrouni et al. (1980) could not detect any change in B cell numbers by radiolabelling small lymphocytes with ^{125}I-anti-IgM during both syngeneic and allogeneic pregnancy. Data about B-cell responses induced by exogenous antigen during pregnancy are also controversial. The primary IgM response to sheep erythrocytes (SRBC) is suppressed according to some authors (Suzuki and Tomasi, 1979) but elevated according to others (Fabris et al., 1973; Mattsson, 1982a; Carter and Dresser, 1983).

These results do not give information about the total activity of the humoral immune system during pregnancy. The total immunoglobulin (Ig) synthesizing activity can be monitored by enumerating the number of Ig-secreting cells within the different lymphoid organs with the protein A plaque assay (Gronowicz et al., 1976). Mattsson (1982b) showed with this method an increase in the number of 'background' Ig-secreting cells in the spleen of syngeneic pregnant mice. A problem with interpreting these results is that such studies do not reveal whether these B cells are internally stimulated or if this change is due to an enhanced reaction to exogenous antigens, e.g. by changed hormone levels.

To exclude exogenous antigens as far as possible we used BALB/c mice which were germfree (GF) reared and fed an ultrafiltered solution of chemically defined (CD) low molecular weight nutrients and were thus devoid of exogenous antigens. We have enumerated the number of 'background' IgM-, IgG- and IgA-secreting cells in the spleen, bone marrow (BM) and mesenteric lymph nodes (MLN) of virgin and pregnant GF-CD mice and compared them with those from virgin and pregnant specific pathogen-free (SPF) and conventionally (CV) raised BALB/c mice given natural ingredient diet (NI).

We also investigated the specificity repertoire of the IgM-secreting cells in the spleen and BM during pregnancy. This was done by using plaque assays detecting cells secreting antibodies of various specificities and determining their frequency among all IgM-secreting cells.

The results show a substantial increase in the numbers of IgM-, IgG- and IgA-secreting cells in spleen, BM and MLN during pregnancy which was most prominent in the GF-CD mice. We did not, however, detect gross changes in the specificity repertoire of the IgM-secreting cells.

Materials and Methods

Mice

BALB/c AnN mice, that were originally obtained from the University of Wisconsin, Madison, WI, USA, were reared and maintained germfree (GF) in the Lobund Laboratory, University of Notre Dame, Notre Dame, IN, U.S.A. and fed

chemically defined ultrafiltered 'antigen-free' diet L489-E14Se and LADEK 69E6 (GF-CD) as described in detail (Pleasants et al., 1985), or specific pathogen-free (SPF) and fed natural ingredient diet L-485 (SPF-NI). Other SPF BALB/c mice were obtained from OLAC 1976 Ltd., Blackthorn, England and Bomholtgard Ltd., Ry, Denmark and were reared and maintained under non-barrier conventional stable conditions and were fed conventional, non-autoclaved diet AMII (CV-NI) (Hope Farm, Woerden, The Netherlands), at the Department of Cell Biology, Immunology and Genetics, Rotterdam, The Netherlands. All animal stocks were originally obtained from the Jackson Laboratory and routinely checked for genetic origin by the supplier. All breeding experiments were performed within 6 mth after obtaining the animals. The conventional BALB/c mice were free of ectoparasites and were routinely checked for their microbiological status. During the experiment *Past. pneumotropica* was found in the nasopharynx of the CV animals. For mating purposes, one BALB/c female mouse was housed with a syngeneic BALB/c male mouse.

Pregnant mice were used at late pregnancy, which was judged by the size of the fetuses. The stage of pregnancy and the number of fetuses was about the same for all groups of pregnant mice. Virgin, age-matched females, similarly fed and maintained, served as controls. It should be mentioned that all mice, including those called 'germfree', carry a latent leukemogenic virus that can be activated by repeated low doses of radiation (Kajima and Pollard, 1968).

Preparation of lymphoid cell suspensions

Spleen, BM and MLN cell suspensions were prepared as described by Benner et al. (1981). The total numbers of nucleated cells were counted with a Coulter Counter, model BZI (Coulter Electronics, Harpenden, Berks, England).

Plaque assays for Ig- and antibody-secreting cells

The target cells for the protein A plaque assay and the antigen-specific plaque assays were prepared, and the plaque assays were performed as described by Hooijkaas et al. (1983). Briefly, nitroiodophenyl (NIP), 4-hydroxy-3,5-dinitrophenyl (NNP) and 2,4,6-trinitrophenyl (TNP) were coupled to sheep red blood cells (SRBC) with 4 or 0.4, 2 or 0.2, or 30 mg, respectively, of the hapten per ml of washed and packed SRBC. These haptenized SRBC are referred to as NIP4-SRBC, NIP0.4-SRBC, NNP2-SRBC, NNP0.2-SRBC and TNP30-SRBC.

Calculation of the total number of Ig-secreting cells and frequency determination of background antigen-specific IgM-secreting cells

The total number of Ig-secreting cells of a given class per organ was calculated by using the number of Ig-secreting cells in the protein A plaque assay and the total cell yield per organ. Ig-secreting cells in the BM were determined in the femoral BM. Following Benner et al. (1981) we have adopted a conversion factor of 7.9 to calculate the number of Ig-secreting cells in the total BM from the number of Ig-secreting cells found in two femurs. The frequencies of background antigen-specific IgM-secreting cells in the spleen and BM were determined in the ap-

propriate plaque assays and calculated as the ratio of the specific IgM-antibody-secreting cells to the total number of IgM-secreting cells.

Statistical analysis

The differences between the experimental groups of mice were evaluated with Student's *t*-test.

Results

Background Ig-secreting cells in spleen, BM and MLN

We have compared virgin and late pregnant germfree mice fed a chemically defined ultrafiltered 'antigen-free' diet (GF-CD) with regard to their number of background Ig-secreting cells in the spleen, bone marrow and mesenteric lymph nodes with two groups of virgin and pregnant control mice that differed in their exogenous antigenic load. One group of barrier maintained SPF BALB/c mice originated directly from the GF-CD mice and were fed autoclaved natural ingredient (SPF-NI), the other group of SPF BALB/c mice came from a different source and were kept under normal stable conditions and fed non-autoclaved natural ingredient (CV-NI).

In the spleen of virgin GF-CD mice the numbers of IgM- and IgG-secreting cells were not different from those of SPF-NI or CV-NI mice, whereas the numbers of IgA-secreting cells were 20-fold lower in GF-CD mice than in virgin CV-NI controls (Table 1). In the spleen of pregnant GF-CD, SPF-NI and CV-NI mice, the total number of IgM-secreting cells was increased 2 to 3 times as compared to their respective virgin controls. The number of IgG-secreting cells did not increase significantly with pregnancy in either group, although we found a higher number in all pregnant mice as compared to their respective virgin controls. However, with regard to IgA-secreting cells, pregnancy had a different effect in CV-NI, SPF-NI and GF-CD mice. In the SPF-NI and CV-NI mice the number of IgA-secreting cells was stable during pregnancy, whereas in GF-CD mice their number increased approximately 4-fold. However, the absolute numbers of IgA-secreting cells were lower in GF-CD mice.

In the BM of virgin GF-CD mice the numbers of IgM-, IgG- and IgA-secreting cells were roughly 3-, 30-, 530-fold reduced, respectively, as compared with virgin CV-NI controls. When compared to virgin SPF-NI mice the numbers of IgM-secreting cells were the same, but the numbers of IgG- and IgA-secreting cells were approximately 10- and 40-fold reduced, respectively. In the BM of pregnant CV-NI, SPF-NI and GF-CD mice the numbers of IgM-secreting cells were 8-, 2- and 5-fold increased, the numbers of IgG-secreting cells 8-, 3- and 11-fold increased and the numbers of IgA-secreting cells 3-, 2- and 40-fold increased, respectively, as compared to their age-matched virgin controls.

In the MLN of virgin GF-CD mice the numbers of IgM-, IgG- and IgA-secreting cells were 44-, 500- and 1000-fold lower than in virgin conventional controls and 22-, 500- and 20-fold lower than in SPF-NI mice, respectively. While the MLN of

TABLE 1

Numbers of background Ig-secreting cells in different lymphoid organs of virgin and pregnant conventional 8–12 wk old BALB/c mice fed natural ingredient (CV-NI), virgin and pregnant specific-pathogen-free 8–12 wk old BALB/c mice fed natural ingredient (SPF-NI) and virgin and pregnant germfree 8–12 wk old BALB/c mice fed chemically defined diet (GF-CD)

Organ	Ig isotype	Ig-secreting cells/organ [a] $\times 10^{-3}$					
		CV-NI		SPF-NI		GF-CD	
		virgin	pregnant	virgin	pregnant	virgin	pregnant
Spleen	IgM	177 ± 46 [a]	500 ± 164 [b]	161 ± 50	494 ± 57 [b]	164 ± 84	383 ± 114 [b]
	IgG	17 ± 11	27 ± 28	13 ± 5	31 ± 12	10 ± 4	28 ± 17 [b]
	IgA	41 ± 20	40 ± 28	8 ± 2	9 ± 4	2 ± 1 [c]	8 ± 3 [b]
BM	IgM	60 ± 22	475 ± 302 [b]	24 ± 3	56 ± 9 [b]	22 ± 5 [c]	99 ± 45 [b]
	IgG	21 ± 6	161 ± 71 [b]	7 ± 5	20 ± 3 [b]	0.7 ± 0.7 [c]	8 ± 4 [b,c]
	IgA	53 ± 12	159 ± 119	4 ± 2	9 ± 0.1 [b]	0.1 ± 0.1 [c]	4 ± 3 [b,c]
MLN	IgM	4 ± 3	5 ± 2	2 ± 4	0.7 ± 0.1	0.09 ± 0.09 [c]	0.16 ± 0.004 [b,c]
	IgG	2 ± 0.9	5 ± 6	2 ± 4	0.2 ± 0.1	0.004 ± 0.009 [c]	0.20 ± 0.15 [b,c]
	IgA	3 ± 2	5 ± 3	0.06 ± 0.08	0.2 ± 0.1	0.003 ± 0.007 [c]	0.15 ± 0.15 [b,c]

[a] Figures represent the arithmetic mean ± SD of 5, 8 and 7 virgin, and 5, 2 and 4 pregnant CV-NI, SPF-NI and GF-CD mice, respectively. In the spleen of virgin and pregnant CV-NI mice 247 ± 73 and 271 ± 93, in the spleen of virgin and pregnant SPF-NI mice 242 ± 65 and 519 ± 116 and in the spleen of virgin and pregnant GF-CD mice 241 ± 69 and 240 ± 57 $\times 10^6$ nucleated cells were found, respectively. The numbers of nucleated cells in the BM were 134 ± 18, 287 ± 35, 223 ± 27, 230 ± 30, 242 ± 87 and 205 ± 70 $\times 10^6$ for virgin and pregnant CV-NI, virgin and pregnant SPF-NI and virgin and pregnant GF-CD mice, respectively. In the MLN their numbers were 40 ± 20, 36 ± 17, 31 ± 14, 25 ± 4, 14 ± 17 and 24 ± 6 $\times 10^6$ for the same groups of mice, respectively.

[b] Significantly different ($P < 0.05$) as compared to the virgin control mice.

[c] Significantly different ($P < 0.005$) as compared to the conventional control mice.

TABLE 2

Relative frequencies of background IgM-secreting cells specific for NIP4-SRBC, NIP0.4-SRBC, NNP2-SRBC, NPP0.2-SRBC, and TNP30-SRBC in spleen and BM of virgin and pregnant conventional BALB/c mice fed natural ingredient (CV-NI), virgin and pregnant SPF BALB/c mice fed autoclaved natural ingredient (SPF-NI) and virgin and pregnant germfree BALB/c mice fed chemically defined diet (GF-CD).

Organ	Antigen	CV-NI		SPF-NI		GF-CD	
		virgin	pregnant	virgin	pregnant	virgin	pregnant
Spleen	NIP4-SRBC	1 in 21 (±4) [a]	1 in 27 (±8)	1 in 30 (±14)	1 in 65 (±10)	1 in 46 (±29)	1 in 56 (±8)
	NIP0.4-SRBC	1 in 188 (±85)	1 in 241 (±78)	1 in 91 (±25)	1 in 286 (±29)	1 in 195 (±131)	1 in 263 (±137)
	NNP2-SRBC	1 in 38 (±11)	1 in 49 (±10)	1 in 58 (±15)	1 in 79 (±10)	1 in 63 (±31)	1 in 64 (±7)
	NNP0.2-SRBC	1 in 1125 (±681)	1 in 812 (±195)	1 in 318 (±236)	1 in 373 (±208)	1 in 284 (±110)	1 in 307 (±173)
	TNP30-SRBC	1 in 122 (±36)	1 in 145 (±90)	1 in 246 (±101)	1 in 428 (±34)	1 in 232 (±71)	1 in 629 (±119) [b]
BM	NIP4-SRBC	1 in 161 (±104)	1 in 57 (±14)	1 in 54 (±14)	1 in 67 (±33)	1 in 111 (±69)	1 in 207 (±193)
	NIP0.4-SRBC	1 in 563 (±198)	1 in 590 (±395)	1 in 201 (±62)	1 in 71 (±4)	1 in 226 (±106)	1 in 416 (±366)
	NNP2-SRBC	1 in 362 (±230)	1 in 169 (±76)	1 in 75 (±28)	1 in 62 (±2)	1 in 60 (±28)	1 in 264 (±210)
	NNP0.2-SRBC	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	TNP30-SRBC	1 in 249 (±127)	1 in 348 (±240)	1 in 176 (±105)	1 in 177 (±27)	1 in 218 (±108)	1 in 560 (±262)

[a] Figures represent the mean ratio of specific IgM-antibody secreting cells to the total number of IgM-secreting cells as detected in the protein A plaque assay. The arithmetic mean ± SD has been calculated.

[b] Significantly different between virgin and pregnant GF-CD mice ($P < 0.001$).

N.D., = not detectable.

the SPF-NI and CV-NI mice showed no significant differences between virgin and pregnant mice, there was a 2-, 5- and 50-fold increase in the number of IgM-, IgG- and IgA-secreting cells in pregnant as compared to virgin GF-CD mice (Table 1).

Frequencies of background antigen-specific IgM-secreting cells in spleen and BM

The frequencies of background antigen-specific IgM-secreting cells were determined in the spleen and BM of virgin and pregnant CV-NI, SPF-NI and GF-CD mice by employing the protein A plaque assay and antigen-specific plaque-assays with the use of a panel of 5 different types of haptenized target SRBC cells. The results given in Table 2 show that in the spleen of virgin and pregnant mice the frequencies of IgM-secreting cells specific for most of the different types of haptenized SRBC tested did not change significantly during pregnancy. This was found for the CV-NI, the SPF-NI and the GF-CD mice. The only significant difference was seen in the frequency of IgM-secreting cells specific for TNP30-SRBC, which was 2-fold lower in pregnant than in virgin GF-CD mice ($P < 0.001$).

In the BM of virgin and pregnant CV-NI and SPF-NI mice the same frequencies of IgM-secreting cells specific for the antigens tested were found as in the BM of GF-CD mice. Thus in the GF-CD mice and the CV-NI and SPF-NI controls a similar proportion of the IgM-secreting cells produce antibodies of the defined specificities, and this remains the same during pregnancy, although the total number of IgM-secreting cells is elevated.

The numbers of cells in the MLN were too low to determine the frequencies of background antigen-specific IgM-secreting cells accurately.

Discussion

Previous studies (Mattsson, 1982b; Carter and Dresser, 1983) have shown an increase in the numbers of IgM- and IgG-secreting cells in the spleen of syngeneically mated CBA mice during pregnancy. We have extended these studies by determining the number of IgM-, IgG- and IgA-secreting cells not only in the spleen but also in the BM and MLN. In the spleen of CV-NI and SPF-NI BALB/c mice a 2-fold increase in the numbers of IgM- and IgG-secreting cells was found in pregnancy, although the difference in the numbers of IgG-secreting cells was not significant. The number of IgA-secreting cells remained essentially the same. The BM showed an increase in the number of Ig-secreting cells of all isotypes, while in the MLN no changes were found.

With conventional and SPF mice it is not clear whether these changes are caused by an altered reaction to exogenous antigens and/or mitogens or by endogenous factors. We have investigated this by using BALB/c mice that were kept under germfree conditions and fed a low molecular weight synthetic diet. These animals show a drastic reduction in the number of IgG- and, especially, IgA-secreting cells in BM and MLN (Table 1). The results show that there is a good correlation between the exogenous antigenic load and the reduction in the number of IgG- and IgA-secreting cells since SPF-NI mice showed intermediate numbers as compared to

both other groups of mice. These results are in good agreement with earlier experiments using the C3H/HeCr strain of mice (Hooijkaas et al., 1984).

During pregnancy in GF-CD mice the numbers of Ig-secreting cells were elevated in all organs tested. The most prominent relative increase was found in the numbers of IgG- and IgA-secreting cells in the BM and MLN. In absolute numbers the increase was, as in the CV-NI and SPF-NI mice, highest among the IgM-secreting cells in the spleen and BM. The increase in the number of Ig-secreting cells in the pregnant GF-CD mice shows that this is not caused by an altered reaction toward exogenous antigens but must have endogenous causes. Changed hormone levels (Fabris et al., 1977), increased erythropoiesis caused by anemia (Mattsson et al., 1982) and antigenic stimulation by embryonic antigens are the most likely causes for these changes.

Allogeneic pregnancy can cause an immunization of the female mouse, as shown by the reported alloantibody levels in the serum (Bell and Billington, 1981). This primary pregnancy-induced alloantibody response is of widely variable titer and has been claimed to lack memory (Smith et al., 1982). However, very few data are available on immunization during syngeneic pregnancy. In order to determine whether the changes we found are permanent, we investigated some multiparous non-pregnant GF-CD, CV-NI and SPF-NI mice that were available in our colony. They showed even higher numbers of IgG- and IgA-secreting cells than the primiparous pregnant mice, which were most clearly seen in the GF-CD mice (data not shown). This suggests that pregnancy induces a new steady state of enhanced immunological activity which remains after termination of the pregnancy.

This new steady state might also induce changes in the specificity repertoire of the Ig-secreting cells. We investigated the specificity repertoire of the IgM-secreting cells by enumerating the frequency of IgM-secreting cells specific for differently haptenized SRBC among all IgM-secreting cells. In the spleen the frequencies of antigen-specific IgM-secreting cells remained the same during pregnancy in the CV-NI, the SPF-NI and in the GF-CD mice (Table 2). In the BM also no significant differences in the specificity repertoire were found, although there was a tendency toward decreased frequencies of the specificities evaluated in the GF-CD mice during pregnancy. Within the limitations of having looked at only a minor part of the specificity repertoire of the IgM-secreting cells, we conclude that no substantial differences occur between virgin and pregnant mice. This suggests a polyclonal activation of the B cell system during pregnancy.

Among the pregnant mice a greater individual variability was found, as can be seen from the standard deviations (Table 2). This might be caused by the individual reaction to the stress that is put upon the immune system in pregnant mice.

The changes we have found in the pregnant mice might influence the B cell development of their offspring. We have investigated this and the results show that the young of GF-CD mothers have the same specificity repertoire of 'background' IgM-secreting cells, although they have a lower rate of development of the 'background' Ig-secreting cells in the spleen (Bos et al., unpublished results).

In this study we did not investigate the specificity repertoire of the IgG- and IgA-secreting cells, which would be of interest because the major numerical changes

were found among these isotypes. Low frequencies of antigen-specific IgG- and IgA-secreting cells are difficult to determine in indirect plaque assays (unpublished observations). It would be of great interest to investigate this aspect in more appropriate assays, such as the ELISA-spot test (Sedgwick and Holt, 1983).

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CHAPTER 7

B CELL REPERTOIRE IN ADULT ANTIGEN-FREE AND CONVENTIONAL NEONATAL BALB/c MICE

I. PREFERENTIAL UTILIZATION OF THE C_H-PROXIMAL V_H GENE FAMILY PC7183

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B cell repertoire in adult antigen-free and conventional neonatal BALB/c mice
I. Preferential utilization of the C_H-proximal V_H gene family PC7183*

Early in ontogeny B cells preferentially use V_H gene families which are most adjacent to the genes coding for the constant part of the immunoglobulin molecule. In conventional adult mice, however, a random usage of V_H gene families has been found. We investigated the role of exogenous antigenic stimulation on this normalization of V_H gene usage by B cells. Therefore, we made use of adult germ-free BALB/c mice fed a chemically defined ultrafiltered antigen-free diet (GF-CD) and neonatal conventional BALB/c mice. Both the adult GF-CD and the newborn conventional mice represent situations with minimal exogenous antigenic stimulation. The results obtained with RNA dot blot hybridization with probes specific for the different V_H gene families showed in hybridomas from adult GF-CD BALB/c mice a preferential usage of the C_H-proximal V_H gene family PC7183. In hybridomas from 5-day-old conventional BALB/c mice a less frequent usage of the J558 V_H gene family was found and an increased usage of the PC7183 V_H gene family than what would be expected from random usage. Evidence is presented that the RNA giving a positive signal with the PC7183 probe represents functional messages for IgM production.

1 Introduction

The V regions of Ig are encoded by germ-line gene segments that are rearranged during B cell development (reviewed in [1]). Three elements encode the V region of the Ig H chain: V_H, D and J_H [2, 3]. Estimates of the number of V_H genes range from 120 [4] to > 1000 [5], and these genes have been classified into nine families on the basis of homology [4, 6, 7]. The V_H genes within a family are highly homologous with > 80% sequence identity, whereas the degree of homology between members of different families ranges from 50% to 70%. The complexity of the families is based on the number of bands hybridizing with V_H-specific probes in Southern blot analysis of genomic DNA [4]. They vary in size ranging from two members in the X24 family to ≥ 60 members in the J558 family. Nucleic acid hybridization studies have indicated that the J558 family may involve at least 500 members [5]. This discrepancy may be due to the presence of many different but related J558-containing fragments in a single Southern band [5].

The V_H genes of the mouse have been mapped on chromosome 12. The V_H gene families are probably organized in overlapping clusters [8]. Although some differences in the chromosomal organization between different inbred mouse strains has been found [9], in general the total number of V_H genes is approximately the same. The various families are located in the following 5′ to 3′ order: 3609-J558-J606-S107-VGAM3.8-3660-X24-Q52-PC7183-D_H as far as currently known from deletion mapping [8–10].

It has been shown that cell lines and hybridomas derived from fetal liver and neonatal spleen early in ontogeny preferentially use the most C_H-proximal V_H gene families [11, 12]. In contrast, in hybridomas derived from LPS-reactive B cells from conventional adult mice there seems to be random usage according to the complexity of the various V_H gene families [13, 14]. The mechanism responsible for this normalization is still unclear. The exposure to exogenous antigens after birth might play a role in this process. This can be studied in germ-free mice that are fed a chemically defined low molecular mass (< 10 kDa) ultrafiltered antigen-free diet (GF-CD). These mice can be kept under these conditions for many generations and are completely devoid of exogenous antigenic stimulation as far as technically achievable at the moment [15].

We have produced hybridomas from LPS-reactive splenic B cells derived from 8–12-week-old GF-CD BALB/c mice and hybridomas from unstimulated splenic B cells derived from 5-day-old conventional BALB/c mice and examined both populations for the expression of V_H gene families in RNA dot blot assays.

2 Materials and methods

2.1 Experimental animals

Germ-free BALB/c mice fed a chemically defined ultrafiltered antigen-free diet L489-E14Se and LADEK 69E6 (GF-CD) were reared and maintained in the Lobund Laboratory, University of Notre Dame, as described in detail [15]. Conventional BALB/c mice were reared and maintained in our own department and fed conventional, nonautoclaved diet AMII (Hope Farm, Woerden, The Netherlands). Conventional Lewis rats were purchased from the Zentralinstitut für Versuchstierzucht, Hannover, FRG.

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Abbreviations: **GF-CD:** Germ-free mice fed chemically defined ultrafiltered antigen-free diet **CV-NEO:** Conventional 5-day-old-mice

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2.2 Hybridoma production

Single-cell suspensions from spleens of 8–12-week-old GF-CD BALB/c mice were obtained as described [16]. After stimulation for 2 days with 50 μg/ml LPS from *E. coli* 026B6 (Difco Laboratories, Detroit, MI), the cells were fused with the SP2/0 myeloma fusion partner at a ratio of 1 : 2 using standard procedures for the production of hybridomas. Fused cells were plated at 5×10^4 cells/well, containing 1×10^6 18 Gy-irradiated rat thymus cells as feeder cells. Spleen cells from 5-day-old conventional BALB/c mice were fused directly with SP2/0 without stimulation with LPS using the same procedure.

2.3 Preparation of RNA, Northern blotting and RNA dot blot assays

Hybridomas were cultured in triplicate to 1×10^5 cells/well and cell lysates were produced as originally described by Manser and Gefter [17]. Briefly, cells were incubated for 30 min with lysis buffer containing 50% DMSO, 0.125% sarcosyl, 12.5 mM sodium citrate (pH 7.0) and 2 M guanidinium isothiocyanate. Hereafter the lysate was spotted directly to nitrocellulose (BA85, Schleicher and Schuell, 's-Hertogenbosch, The Netherlands) using the MinifoldII (Schleicher and Schuell) slot blot apparatus. Purified RNA (10–50 μg) was obtained by precipitation of at least 10^7 hybridoma cells in 3 M LiCl, 6 M urea. Aliquots of 1, 0.5 and 0.25 μg purified RNA was spotted to nitrocellulose in 20 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 6.5). For Northern blotting 20 μg RNA was electrophoresed in 2.2 M formaldehyde, 1% agarose gels and blotted to nitrocellulose.

2.4 Hybridization of nitrocellulose filters

Filters were hybridized with ^{32}P-labeled DNA probes in hybridization mixture containing 10 × Denhardt's solution [2% Ficoll (Pharmacia, Uppsala, Sweden), 2% BSA, 2% polyvinylpyrrolidone], 50% formamide, 10% (w/w) dextran sulfate, 1 M NaCl, 50 mM Tris-HCl (pH 7.5) and 100 μg/ml denatured salmon sperm DNA for 18 h at 42 °C. Filters were subsequently washed twice in 3 × SSC, 0.1% SDS and twice in 1 × SSC, 0.1% SDS at 42 °C prior to autoradiography.

2.5 DNA probes

The following probes were used in this study. The C_μ probe is a 900-bp Pst I fragment derived from a cDNA clone (pABu-11) containing the C_μ-coding regions [18]. The VNPB4 probe is a 315-bp Eco RI/Pst I fragment containing the V segment corresponding to a genomically unrearranged V_H558 gene segment [19]. The V_H81X probe is a 280-bp Eco RI/Pst I fragment isolated from a genomic library [11]. The V_H Q52 probe is a 300-bp Bam HI/Eco RI fragment isolated from the myeloma Q52 and contains the sequence spanning from codon 14 to 24 bp of the J_H1 segment [4]. The pBV14 J606 probe is a 600-bp Bam HI/Eco RI fragment isolated from a genomic library [4]. The P6.3RI probe is a 600-bp XbaI/Eco RI fragment cloned from a cDNA library derived from the hybridoma LB8. The V_H segment contained in this fragment shows a 89% homology to the germ-line MOPC460 sequence, while J sequences are not included. The 38C probe is a 800-bp Bam HI/Eco RI fragment isolated from the B cell lymphoma 38C-13 [20]. The V_HX-24 probe is a 500-bp EcoRI fragment derived from a genomic library [21]. The V_H23-9 probe is a 500-bp Bam HI/Pst I fragment containing the V_H sequence belonging to the V_H 3609 family. These probes were kindly provided by Drs D. Holmberg, J. Kearney, F. Alt and D. Schulze.

2.6 Isotype determination

Hybridoma SN were screened for isotype production using a sandwich ELISA with affinity-purified rat anti mouse κ mAb 226 [16] as first antibody and affinity-purified isotype-specific goat anti-mouse H chain antisera coupled to alkaline phosphatase (AP; Southern Biotechnology Associates, Birmingham, AL) as detecting antibody.

3 Results

3.1 Production of hybridomas

For the production of hybridomas, spleen cells from 8–12-week-old GF-CD BALB/c mice were polyclonally stimulated with LPS *in vitro* for 2 days. Thereafter the cells were fused with SP2/0. The cells were plated at such a density that growth occurred in 15%–40% of the wells. In four independent fusions we established 490 hybridomas, of which 421 produced IgM, κ (86%) and one hybridoma produced IgG_3, κ. In the remaining 68 hybridomas no Ig production was detected. In four independent fusions 335 hybridomas from unstimulated spleen cells from 5-day-old conventional (CV-NEO) BALB/c mice were generated. From these hybridomas 89 produced IgM and no other isotypes were found among the other hybridomas.

3.2 Determination of V_H gene family usage in lysates of GF-CD hybridomas

As initial screening of the V_H gene usage we used the direct lysate method described by Manser and Gefter [17]. Therefore at least 1×10^5 hybridoma cells were plated in triplicate in 96-well plates. Thereafter cells were lysed with a lysate buffer, using 2 M guanidinium isothiocyanate as chaotropic salt used to preserve RNA. Subsequently, the lysate was directly spotted to nitrocellulose.

The nitrocellulose filters were hybridized to probes specific for the V_H gene families J558, PC7183, Q52, J606, 36–60 and S107 and to the C_μ probe (Table 1). Among the C_μ^+ lysates 43% did not react with any of the V_H gene probes used. A possible explanation might be that they belong to one of the other V_H gene families that were not tested here. Alternatively, it might be that this direct lysate method does not yield enough specific RNA for a positive signal with the V_H probes used.

Among the V_H^+ hybridomas 16 reacted with more than one probe and could not be assigned to a single V_H gene family. A possible explanation might be that these hybridomas were not monoclonal when tested, although the fusion efficiency and the time that they were cultured predicts only a few multiple clones.

Among the 18 hybridomas that could be appointed to a single V_H gene family a correlation was found between the complex-

Table 1. V_H gene expression determined in cell lysates from hybridomas derived from LPS-reactive B cells of adult GF-CD BALB/c mice

a) The size of a V_H gene family was determined from the number of hybridizing bands in a Southern blot [4].
b) Number of hybridomas belonging to a V_H gene family as determined by hybridization of cell lysates to the different probes.

ity of the various V_H gene families and the percentage of usage of these families, except for the V_H gene family PC7183, which was used by 28% of the V_H^+ hybridomas, which is approximately three times higher than what is expected from the complexity of this V_H gene family. Because of the high level of uncertainty in these initial screenings we decided to purify RNA from a number of hybridomas from adult GF-CD mice ("GF-CD hybridomas") and from hybridomas derived from newborn conventional mice ("CV-NEO hybridomas").

3.3 Determination of V_H gene usage with purified RNA from GF-CD and CV-NEO hybridomas

Fifty-nine GF-CD and 207 CV-NEO hybridomas were grown to more than 1×10^7 cells. The CV-NEO hybridomas were chosen at random from the 335 growing hybridomas before they were screened for Ig production, while from the GF-CD hybridomas only IgM-producing hybridomas were used. RNA was purified from these hybridomas by LiCl precipitation. This yielded 10–50 μg RNA. Aliquots of 1, 0.5 and 0.25 μg were spotted to nitrocellulose. Hybridomas were screened with the C_μ probe. This resulted in 65 C_μ^+ CV-NEO and 59 C_μ^+ GF-CD hybridomas that could be screened for V_H gene usage. Fig. 1 shows an example of the subsequent hybridization to the different V_H gene probes from a number of CV-NEO hybridomas. Hybridomas were assigned to a particular V_H gene family if they gave a clear signal with the C_μ probe and only a single V_H gene family probe (see section 3.4).

The distribution of the GF-CD and CV-NEO hybridomas among the different V_H gene families in relation to the size and position of those V_H gene families is shown in Fig. 2. In both hybridoma panels the PC7183 V_H gene family was preferentially used. This was most clear among the CV-NEO hybridomas where 43% of all C_μ^+ hybridomas used this V_H gene family, while this figure was only 27% among the hybridomas from adult GF-CD mice. The large J558 V_H gene family was used in 44% of the GF-CD hybridomas which is in accordance with the size of this family. On the other hand, only 23% of the CV-NEO hybridomas used this gene family, which is lower than expected. There were eight GF-CD and

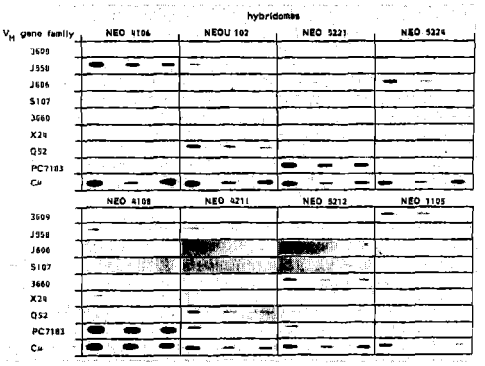

Figure 1. Dot-blot hybridization of different CV-NEO hybridomas to probes specific for the different V_H gene families. Aliquots of 1, 0.5 and 0.25 μg of purified RNA were dot blotted to nitrocellulose and hybridized to the C_μ probe and to probes specific for the different V_H gene families.

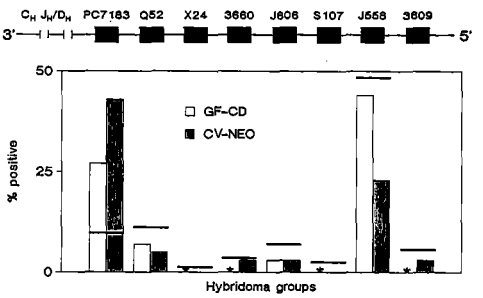

Figure 2. V_H gene family usage in GF-CD and CV-NEO hybridomas compared to the size and position of the V_H gene families. GF-CD (59) and CV-NEO (65) hybridomas were tested for V_H gene family usage by hybridization of purified RNA to the different probes. Eleven GF-CD and 13 CV-NEO hybridomas could not be assigned to one V_H gene family. The horizontal line within each V_H gene family represents the expected percentage according to the size of that V_H gene family. The chromosomal order of the V_H gene families is shown at the top of the figure. * = not done.

two CV-NEO C_μ^+ hybridomas that gave positive signals with more than one V_H gene family. Furthermore, 3 C_μ^+ GF-CD hybridomas and 11 C_μ^+ CV-NEO hybridomas did not give a positive signal with any of the V_H probes used. Thus, V_H gene analysis of purified RNA enabled determination of the V_H gene family usage in 80% of the GF-CD and the CV-NEO hybridomas, which is substantially higher than the 49% resolution using the Manser/Gefter [17] technique. Despite this difference in resolution, both assays point to a preferential usage of the PC7183 V_H gene family in both hybridoma panels tested. The J558 V_H gene family was underrepresented among the CV-NEO hybridomas, while this was not the case among the GF-CD hybridomas.

3.4 Northern blotting analysis of CV-NEO hybridomas giving a positive signal with the PC7183-specific probe

In order to investigate whether the PC7183 V_H gene expression represents functional messages we performed Northern blotting analysis on a number of CV-NEO hybridomas that were positive with the V_H81X probe which is specific for the V_H gene family PC7183. Some of them also reacted with the C_μ probe and some of them did not. As can be seen in Fig. 3, a functional message of approximately 2.4 kb was only seen when there was a positive signal in the RNA dot-blot assay for both probes. As a negative control the TNP2-1 hybridoma was included which is C_μ^+, but utilizes the J558 gene family. The 2.4-kb message suggests that the V_H81X hybridizing signal is derived from the functional message for the production of IgM. In the case of the C_μ^+ hybridomas no V_H81X$^+$ signal was found on Northern blots. Therefore only hybridomas that were positive for C_μ and one of the V_H gene family probes were considered positive in the RNA dot blot assay (see Sect. 3.3).

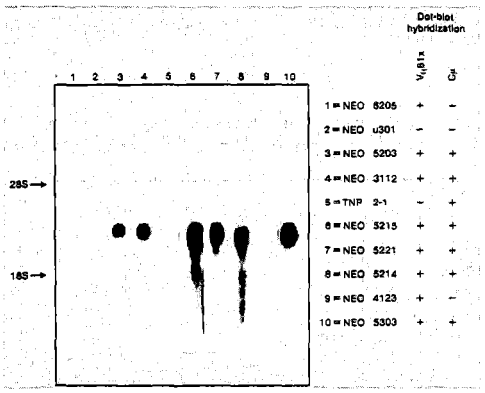

Figure 3. Northern blot hybridization of hybridoma RNA to the V_H81X probe. Purified RNA (20 µg) was electrophoresed, blotted to nitrocellulose and hybridized to the V_H81X probe, which is specific for the PC7183 V_H gene family. For comparison the results of the RNA dot blot assay are given.

4 Discussion

Several studies with different experimental approaches have demonstrated that V_H usage is biased towards C_H-proximal V_H segments in cell lines derived from fetal or newborn mice [11, 12, 14, 22], while V_H usage is more normalized – that is, more related to family size instead of family position – in hybridomas and spleen cultures, or LPS-stimulated splenocytes derived from adult spleen [13, 23, 24]. A recent study [25] has verified that V_H usage follows this pattern *in vivo* by demonstrating that V_H family usage in the newborn liver is dependent on both family position and size, whereas in both unstimulated and LPS-stimulated adult splenocytes V_H family usage is primarily dependent on family size. These studies have raised the question of when, where and why normalization of V_H gene usage occurs in the transition from neonate to adult. Selective (exogenous) antigenic forces might play a role in this process.

Among hybridomas derived from 5-day-old conventional BALB/c mice we found that 43% utilized the C_H-proximal V_H gene family PC7183 and 23% utilized the J558 V_H gene family, which indeed points towards a position-dependent family usage instead of a family size-dependent usage. This is in agreement with an earlier report [14], although Holmberg et al. found the C_H-proximal V_H family Q52 (together with PC7183) preferentially used. However, when the data of these two C_H-proximal families, which are located intermingled on chromosome 12 [26], are combined 48% usage of these C_H-proximal V_H gene families is found. Recently Glotz et al. [27] investigated hybridomas from 1-day-old neonatal BALB/c mice. These mostly IgG_{2b}-producing hybridomas showed a family size-dependent pattern of V_H gene family usage, although PC7183 was slightly overrepresented [27]. A number of studies have shown that hybridomas from conventional adult C57BL/6 [13], A/J [28] and BALB/c mice [14] display a family size-dependent usage of V_H gene families. We show here with two different methods that in hybridomas derived from LPS-reactive B cells from adult GF-CD BALB/c mice 27%–28% utilized the V_H gene family PC7183, which is 2.7 times higher than what is expected from the size of this V_H gene family. In this respect adult GF-CD mice resemble conventional neonatal mice and differ from adult conventional mice. If exogenous antigenic stimulation is avoided during lifetime, clonal selection will hardly occur. Therefore, the usage of V_H genes will be more comparable to that of the neonatal B cell pool. In adult GF-CD mice, however, besides a higher percentage of B cells expressing the PC7183 V_H gene family, there is a higher percentage of B cells expressing the J558 family compared to neonatal B cells. This suggests that normalization has taken place to some extent in GF-CD mice. This could be caused by some endogenous antigenic stimulation, as suggested by the rise of the number of background IgG- and IgA-secreting cells in maturing GF-CD mice [29].

The mechanism by which exogenous antigenic stimulation can influence the expression of V_H gene remains unclear. The V-D-J rearrangement obviously takes place before the Ig molecule can serve as an antigen receptor on the membrane. Transcription of germ-line V_H genes might be a target for regulation [19].

The dynamics of B cell production also need to be considered. Exogenous antigenic stimulation elevates the production rate of B lymphocytes in the BM [30]. Preferential usage of 3′ V_H genes has been found in pre B cell lines derived from adult BM [11]. From the large number of newly formed B cells in the BM only a small fraction is incorporated in the peripheral B cell pool [31]. This transition could be prone to selection mechanisms, where the large spectrum of exogenous antigens could select for the largest possible diversification of the expressed germ-line V_H gene repertoire and may cause clonal development of the B cells with high-affinity receptors. It remains to be established whether this selection is performed directly by exogenous antigens or by idiotype-anti-idiotype interactions which reflect the interactions of peripheral B cells with the exogenous antigens.

The availability of hybridoma collections derived from conventional neonatal spleen and from LPS-reactive B cells from adult GF-CD mice enables investigations of the relationship between the usage of the V_H gene family and the antigen specificity of the secreted Ig without interference of the environment. In the following report [32] we show that the high

frequency of multireactive antibodies found among neonatal hybridomas which preferentially utilize the more 3′ V_H gene families [33] also occurs among GF-CD hybridomas.

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CHAPTER 8

B CELL REPERTOIRE IN ADULT ANTIGEN FREE AND CONVENTIONAL NEONATAL BALB/c MICE
II. ANALYSIS OF ANTIGEN-BINDING CAPACITIES IN RELATION TO V_H GENE USAGE

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B cell repertoire in adult antigen-free and conventional neonatal BALB/c mice
II. Analysis of antigen-binding capacities in relation to V_H gene usage*

Hybridomas were derived from lipopolysaccharide-reactive splenic B cells of adult germ-free BALB/c mice fed a chemically defined ultrafiltered "antigen-free" diet (GF-CD) and from splenic B cells of 5-day-old conventional (CV-NEO) BALB/c mice. The monoclonal antibodies (mAb) from both collections of hybridomas were tested for reactivity against a large panel of antigens of exogenous and endogenous origin. As a source of natural exogenous antigens 36 different bacteria and 9 different viruses were used, while as endogenous antigens frozen tissue sections of stomach, liver and kidney, the Hep-2 cell line and the anti-idiotopic mAb Ac38 and Ac146 were used. In both collections of mAb approximately 70% reacted with one or more bacterial antigens, while no reactivity could be detected against the viral antigens. Of the GF-CD and CV-NEO hybridomas, 16% and 19%, respectively, reacted with one or more frozen tissue sections. Overall 56% and 68% of the GF-CD and CV-NEO hybridomas, respectively, were producing multireactive antibodies reactive to several exogenous and/or endogenous antigens.
Among the GF-CD hybridomas a correlation was found between multireactivity and the usage of the V_H gene family PC7183. In CV-NEO hybridomas, however, the preferential utilization of the V_H gene family PC7183 was found among both mono- and multireactive hybridomas. The results suggest (a) that the actual B cell repertoire of neonatal mice consists of a large proportion of multireactive B cells which are reactive with autoantigens and bacterial antigens, but not viral antigens and (b) that in antigen-deprived mice the neonatal repertoire is largely preserved during maturation of the mice.

1 Introduction

Early in ontogeny a high frequency of B cells are found which can bind to multiple antigens, among which autoantigens [1]. For example from neonatal spleen many autoreactive hybridomas have been isolated [1]. This has been correlated with preferential utilization of V_H genes which are most proximate to the genes coding for the C part of the H chain, namely Q52 and PC7183 [2, 3]. In conventional adult mice these multireactive B cells are much less frequent [4] coinciding with a random usage of V_H genes [5–7]. Among hybridomas derived from LPS-reactive B cells from adult A/J mice a high proportion of autoreactive hybridomas was found. However, only a few of them were multireactive [8]. Apparently the B cell repertoire changes during lifetime, presumably due to natural exogenous antigenic stimulation causing clonal selection and diversification (reviewed in [9]).

The influence of natural exogenous antigenic stimulation can be well studied in germ-free mice fed a chemically defined low molecular mass (< 10 kDa) ultrafiltered antigen-free diet (GF-CD). These mice can be kept under these conditions for many generations and are completely devoid of exogenous antigenic stimulation as far as technically achievable at the moment [10]. We have produced a panel of hybridomas from the LPS-reactive splenic B cells from such BALB/c mice as well as from 5-day-old conventional (CV-NEO) BALB/c mice.

In the preceding report [11] we have reported that hybridomas derived from adult GF-CD mice as well as from CV-NEO mice preferentially use the same V_H gene family, namely PC7183. This raised the question whether the B cell repertoire of GF-CD mice has similarities with the B cell repertoire of neonatal mice. Therefore, we tested both collections of hybridomas for reactivity towards a large number of exogenous and endogenous antigens. As exogenous antigens a broad panel of bacteria and viruses were used, while as endogenous antigens tissue sections, acetylcholine receptor and anti-idiotopic mAb were tested. The reactivity toward these antigens was compared with the usage of the V_H gene families in these hybridomas.

2 Materials and methods

2.1 Hybridoma collections and antigens

The origin and production of hybridoma collections from GF-CD and CV-NEO BALB/c mice have been described in the preceding report [11]. A list of the different antigens and the hybridomas tested is shown in Table 1. Anaerobic bacterium strains, originally isolated from human faeces, were kindly provided by Dr M. P. Hazenberg from our department. Vesicular stomatitis virus (VSV) reactivity was tested by Dr R.

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Abbreviations: **GF-GD:** Germ-free mice fed chemically defined ultrafiltered antigen-free diet **CV-NEO:** Conventional 5-day-old mice

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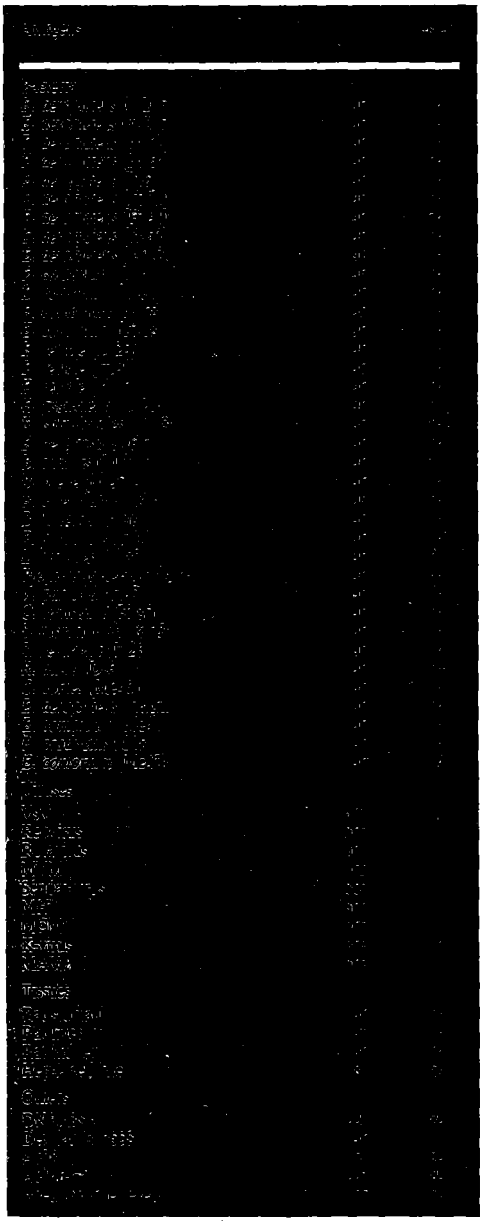
Table 1. List of antigens and number of hybridomas tested



R. Zinkernagel (Institute of Pathology, Zürich, Switzerland), while reactivity to the other viral strains was tested by Dr A. Osterhaus (National Institute for Public Health and Environmental Protection, Bilthoven, The Netherlands). Frozen tissue sections of rat stomach, liver and kidney and cytocentrifuge preparations of the Hep-2 cell line were kindly provided by Dr H. Hooijkaas of our department. DNP was coupled to BSA at a ratio of 27:1 as described previously [12]. Dextran B1355S was tested by Dr E. Kölsch (University of Münster, FRG). Ac38 and Ac146 mAb ([13]; kindly provided by Dr. K. Rajewsky, Institute of Genetics, Köln, FRG) were affinity purified on a protein A-Sepharose column. Purified acetylcholine receptor from *Torpedo californa* was kindly provided by Dr F. G. C. M. UytdeHaag (National Institute for Public Health and Environmental Protection, Bilthoven, The Netherlands).

2.2 Hemagglutination (HA) assay and ELISA

Anti-dextran B1355S reactivity was determined by HA as described previously [14]. Sandwich ELISA was performed as described before [11]. A signal was considered positive if the absorbance at 405 nm was higher than the signal of the negative control + 3 SD. For determining the IgM concentration, affinity-purified rat-anti mouse IgM mAb (R33, 10 μg/ml) was used as first antibody. Absolute concentration was determined by comparing with a standard concentration of affinity-purified monoclonal IgM (Sp603). For determination of idiotope-positive hybridomas mouse anti-idiotopes mAb Ac38 and Ac146 were used as the first step. $DNP_{27}BSA$ and purified acetylcholine receptor were used as first step at 10 and 1 μg/ml, respectively.

2.3 Immunofluorescence assay

Anaerobic bacteria suspended in PBS were coated on glass slides by air drying and fixed by three quick exposures to a flame. These preparations as well as frozen tissue sections of rat stomach, kidney and liver, and cytocentrifuge preparations of the Hep-2 cell line were incubated with hybridoma SN for 1 h at room temperature. After washing with PBS plus 0.5% BSA, slides were incubated with appropriately diluted goat anti-mouse Ig coupled to FITC (Nordic Laboratories, Tilburg, The Netherlands). Slides were examined with a Zeiss (Oberkochen, FRG) epi-fluorescence microscope.

2.4 Anti-viral reactivity

Fivefold diluted hybridoma SN were screened in ELISA against reovirus, rotavirus, minute virus of mouse (MVM), Sendai virus, mouse cytomegalovirus (MCMV), mouse hepatitis virus (MHV) and mouse adenovirus (MAV). For confirmation of the results from the ELISA, some SN were also screened in an immunofluorescence assay. VSV was tested in a neutralization assay of 70–100 pfu of either VSV-Indiana or VSV-New Jersey as described elsewhere [15].

3 Results

3.1 Hybridoma collections

The production of the hybridoma collections has been described in the preceding report [11]. From adult GF-CD and CV-NEO mice, collections of 421 and 89 IgM, κ hybridomas

were isolated, respectively. From these collections a number of randomly chosen hybridomas were tested in the different assays.

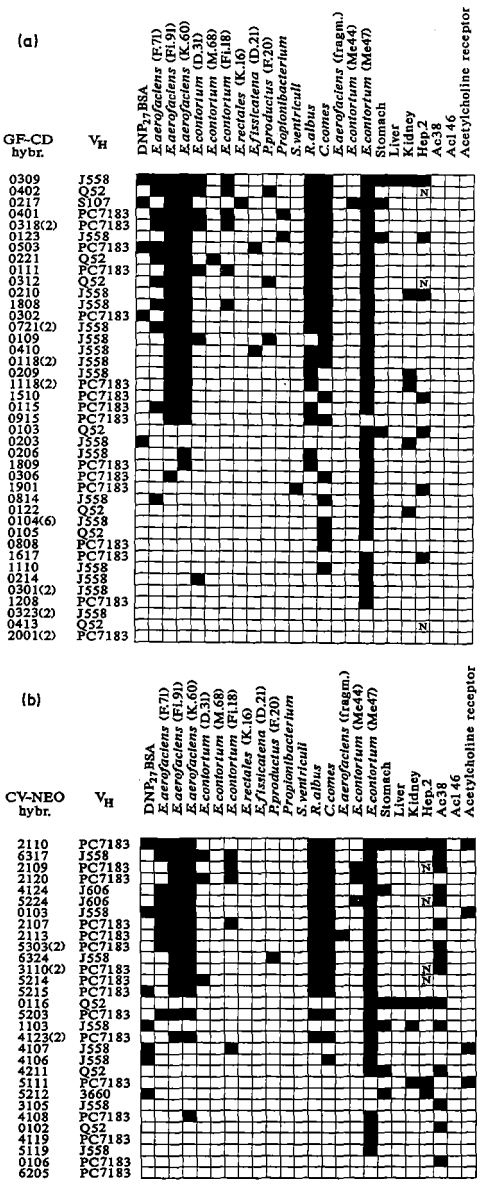


Figure 1. Reactivity pattern and V_H gene usage of different GF-CD hybridomas (A) and CV-NEO hybridomas (B). Closed squares represent positive reactions; open squares represent negative reactions; N = not done.

3.2 Reactivity towards exogenous antigens

3.2.1 Bacteria

Of the GF-CD and CV-NEO hybridomas 74% and 68%, respectively, reacted with one or more of the 36 bacterial strains tested. Among the hybridomas that reacted with more than one strain, unique reactivity patterns were seen (Fig. 1). From the hybridomas that reacted with more than one bacterial strain 26/30 and 17/20 different patterns seen were unique for a certain GF-CD or CV-NEO hybridoma, respectively. With 19 strains no reactivity was found among the GF-CD and CV-NEO hybridomas tested and for convenience they are not shown in Fig. 1. Some bacteria (like *C. comes* and *R. albus*) have antigens that are recognized by a large number of hybridomas, while others (*e.g., E. rectales* and *P. productus*) show reactivity with a few hybridomas only. The two different bacterial strains of *E. aerofaciens* Fi.91 and K.60 were differentially recognized by the hybridomas GF-CD 0206 and GF-CD 0306. *E. aerofaciens* fragments were only recognized by CV-NEO 2113. Affinity-purified IgM of hybridoma CV-NEO 2110 showed exactly the same reactivity pattern as the original SN.

3.2.2 Viruses

In contrast with the high number of GF-CD and CV-NEO hybridomas that reacted with different bacteria, none of the GF-CD hybridomas reacted with the viruses tested (Table 1). CV-NEO hybridomas were not tested for viral reactivity.

3.2.3 Others

Other exogenous antigens that were tested were DNP_{27}BSA and dextran B1355S. We found 18 out of 220 (8%) GF-CD and 12 out of 89 (13%) CV-NEO hybridomas that reacted with DNP_{27}BSA. Reactivity with dextran B1355S was not found among the 96 GF-CD hybridomas tested. Reactivity with dextran B1355S was not tested among the CV-NEO hybridomas.

3.3 Reactivity towards endogenous antigens

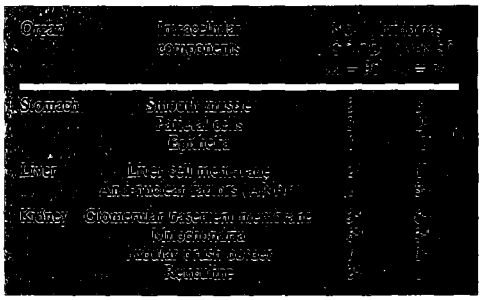
3.3.1 Frozen tissue sections of stomach, kidney and liver

Altogether, 18 out of 96 (19%) GF-CD and 15 out of 74 (20%) CV-NEO hybridomas reacted with rat stomach, kidney, liver and/or the Hep-2 cell line. In the organs several intracellular components were stained (Table 2). From the positively reacting hybridomas 13/18 (72%) GF-CD and 7/15 (46%) CV-NEO hybridomas reacted with only one intracellular component. The results in Table 2 show that a similar proportion of hybridomas in both panels reacted with the various intracellular components.

3.3.2 Other antigens

Altogether, 42 out of 89 (47%) CV-NEO hybridomas reacted with the anti-idiotopic mAb Ac38, which is higher than what was found among GF-CD hybridomas (6/260 = 2%). None of the $Ac38^+$ GF-CD hybridomas and only 5/42 $Ac38^+$ CV-NEO hybridomas reacted with DNP, which is the hapten recognized

Table 2. Reactivity of GF-CD and CV-NEO hybridomas with frozen tissue sections of rat stomach, liver and kidney



a) Five GF-CD and seven CV-NEO hybridomas were reactive with multiple intracellular components.
b) ANF was also detected on the Hep-2 cell line.

by the original idiotope-bearing mAb B1–8. The idiotope recognized by the anti-idiotopic antibody Ac146 was much less frequently expressed in both populations (1/223 GF-CD and 0/89 CV-NEO hybridomas). The acetylcholine receptor was recognized by 6/80 (8%) CV-NEO hybridomas and 4/155 (3%) GF-CD hybridomas.

3.4 Multireactivity

In both populations we found a high proportion of hybridomas that reacted with more than one antigen. These hybridomas are referred to as multireactive. Multireactivity ranged from 2 to 12 different antigens (Fig. 2). We have ranked the hybridomas from high to low multireactivity in Fig. 1. No significant differences were found in IgM concentration between high and low multireactive antibodies (83 ± 25 and 81 ± 25 μg/ml for high and low multireactive CV-NEO hybridomas, respectively and 42 ± 12 and 30 ± 10 μg/ml for high and low multireactive GF-CD hybridomas, respectively). When the reactivity against endogenous and exogenous antigens was compared in both groups, the CV-NEO hybridomas showed a higher proportion of hybridomas that were reactive with

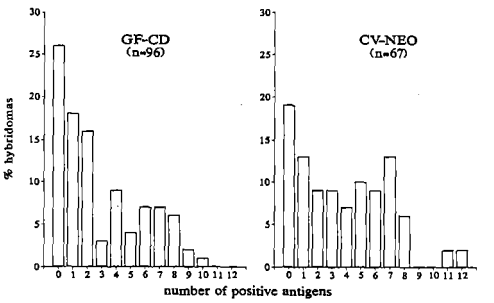


Figure 2. Percentage of GF-CD (left) and CV-NEO (right) hybridomas reactive with different numbers of antigens.

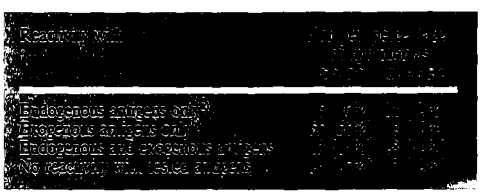
endogenous antigens (Table 3). This was mainly caused by the higher number of CV-NEO hybridomas that were Ac38$^+$. In both populations we have found hybridomas that were reactive with either exogenous or endogenous antigens and a group of hybridomas that reacted with endogenous as well as exogenous antigens (Table 3).

3.5 V_H gene usage

For 55 of the above GF-CD hybridomas and 33 of the above CV-NEO hybridomas V_H gene family usage has been determined [11]. A preferential utilization of the V_H gene family PC7183 was found in both groups of hybridomas. The large J558 V_H gene family which comprises approximately 50% of the V_H genes as determined by Southern blotting analysis [16], was used by 49% of the GF-CD and 26% of the CV-NEO hybridomas.

In order to investigate the relationship between the preferential utilization of the PC7183 V_H gene family and the multireactivity of the hybridomas, both groups of hybridomas were divided into mono- and multireactive hybridomas and the V_H gene usage of these groups was compared. As shown in Fig. 3, the preferential utilization of PC7183 among GF-CD hybridomas was mainly found among multireactive hybridomas, while the V_H gene families J558 and Q52 seemed

Table 3. Reactivity of GF-CD and CV-NEO hybridomas with endogenous and exogenous antigens



a) The endogenous antigens tested were frozen tissue sections of rat stomach, kidney and liver, acetylcholine receptor and the idiotopes Ac38 and Ac146.
b) The exogenous antigens tested were a series of anaerobic bacteria and $DNP_{27}BSA$.

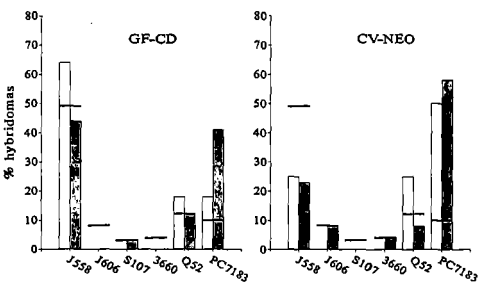


Figure 3. Percentage of mono- (left bars) and multireactive (right bars) hybridomas that utilize various V_H gene families. The horizontal line within each V_H gene family represents the expected percentage of hybridomas according to the size of that V_H gene family [17].

to be used according to their size. The frequency of the other V_H gene families was too low to interpret. In the group of CV-NEO hybridomas the higher percentage of PC7183 and the lower percentage of J558 was found equally in the subgroups mono- and multireactive hybridomas. The other V_H gene families were used according to their family size. No clear correlation was found between reactivity against exogenous or endogenous antigens and the usage of a particular V_H gene family.

4 Discussion

It has been suggested that the preimmune B cell repertoire, as determined by hybridomas from LPS-reactive B cells of conventional mice, has a bias towards autoantigens [8]. B cells with the capacity of secreting autoantibodies have been shown to be components of the normal immune system. Normal human serum contains antibodies against neurofilaments, tubulin, actin, transferrin, thyroglobulin etc. [17]. Autoreactive B cells can be isolated from spleens of adult conventional [18, 19], neonatal [1], and adult germ-free mice [20]. However, reactivity against autoantigens and foreign antigens depends very much on the number and quality of the antigens tested. Therefore it is difficult to determine a bias towards either endogenous or exogenous antigens. We show here that within a panel of 74 randomly chosen hybridomas derived from the spleen of 5-day-old conventional BALB/c mice there is at least as much reactivity against bacterial antigens as against intracellular components. This reactivity is mainly caused by hybridomas that react with multiple antigens, both from endogenous and exogenous origin.

All our neonatal hybridomas produced IgM. This is in agreement with data from others [1–3]. In contrast, Glotz found mostly IgG_{2b}-producing hybridomas after fusion of 1-day-old BALB/c spleen cells [21]. They suggested that the age of the newborn mice might be important, because after fusing spleen cells of 8-day-old mice 60% of the hybridomas produced IgM, while the others produced other isotypes. Among their hybridomas a high frequency of autoreactive hybridomas were found, but reactivity against foreign antigens was also detected. Multireactive hybridomas were found in a higher frequency among neonatal hybridomas [1] than among hybridomas derived from the spleen of conventional adult mice [4]. We show here that among hybridomas derived from LPS-reactive splenic B cells from GF-CD BALB/c mice, there is also a high frequency (56%) of multireactivity.

Autoreactive hybridomas have also been isolated from unstimulated spleen cells from conventional adult mice [17]. Whether these autoreactive antibodies can also react against exogenous antigens has not been investigated. However, Monestier et al. [22] have shown cross-reactivity between autoantigens and foreign antigens for a number of hybridomas. Probably, B cells that produce multireactive, including autoreactive, antibodies persist throughout the mouse lifetime, but their frequency seems to be higher early in ontogeny. This conversion, however, is much less prominent in the absence of exogenous antigenic stimulation as shown in GF-CD mice. This indicates that exogenous antigenic stimulation plays an important role in the development of the actual B cell repertoire.

The physiological role of the multireactive B cells is still a matter of speculation. It is suggested that these cells are involved in an early idiotype network [23]. Another possible function of these multireactive antibodies early in ontogeny may be a first line of defence which can react, probably with low affinity, with a broad spectrum of antigenic structures [24]. It is remarkable that this neonatal germ-line repertoire of multireactive B cells consisted of antibodies which reacted with many bacterial antigens and not with the viral antigens tested. With the restrictions that we have analyzed only a limited panel of bacterial and viral antigens, this may suggest an evolutionary selection of V_H genes which can react with bacterial antigens.

In cell lines and hybridomas from fetal and neonatal mice a preferential utilization of the most C_H-proximal V_H gene families has been observed [2, 3, 25], while in conventional adult mice a random usage is found [5–7]. This has been correlated with the occurrence of multireactive, autoreactive antibodies [2]. Adult GF-CD mice, however, also show a preferential utilization of the 3′ V_H gene family PC7183. In this report we show that in GF-CD mice PC7183 is also correlated with multireactive antibodies. This is in contrast with the CV-NEO hybridomas, where the usage of J558 was lower and of PC7183 higher than what is expected from a random usage, both in mono- and multireactive hybridomas.

The normalization of the usage of the J558 V_H gene family found in GF-CD hybridomas, especially among monoreactive hybridomas, suggests that some development of the $J558^+$ B cell repertoire has taken place in GF-CD mice. This might be caused by endogenous antigenic stimulation, as suggested by the rise of the number of background IgG- and IgA-secreting cells in maturing GF-CD mice [26]. Therefore, it will be interesting to investigate whether the B cell repertoire of adult GF-CD mice completely normalizes after putting such mice back into a normal environment.

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CHAPTER 9

GENERAL DISCUSSION

9.1. B cell repertoires in 'antigen-free' mice

In this thesis the influence of the exogenous antigenic environment on the murine B cell repertoire has been studied. This was done by using neonatal and adult germfree BALB/c mice fed a chemically defined ultrafiltered 'antigen-free' diet (GF-CD mice) and conventional syngeneic control mice fed natural ingredient diet (CV-NI mice). The actual B cell repertoire was studied by enumerating the total numbers of Ig-secreting cells and the frequencies of antigen-specific Ig-secreting cells and by analysis of the serum Ig levels.

In neonatal mice the development of the compartment of Ig-secreting cells in the spleen was faster in CV-NI mice than in GF-CD mice. During this development the frequency of antigen-specific IgM-secreting cells did not change in both groups of mice (Chapter 3).

At the adult age the total numbers of IgM-secreting cells, frequencies of antigen-specific IgM-secreting cells and serum IgM levels did not differ between GF-CD and CV-NI mice. However, total numbers of IgG- and IgA-secreting cells and serum IgG and IgA levels were severely reduced in GF-CD mice compared to CV-NI mice. Also the frequency of antigen-specific IgG- and IgA-secreting cells did differ between adult GF-CD and CV-NI mice (Chapters 3, 4 and 5). This data suggests that the actual IgM specificity repertoire is established independent of exogenous antigenic stimulation, while the actual IgG and IgA specificity repertoires are highly influenced by exogenous antigens.

During pregnancy a temporary raise in the numbers of IgG- and IgA-secreting cells was found in GF-CD mice, suggesting some influence of endogenous factors (hormones or fetal antigens) on the actual IgG and IgA specificity repertoires (Chapter 6).

The available repertoire was studied by the determination of the usage of V_H gene families and the reactivity against a panel of exogenous and endogenous antigens in collections of hybridomas of LPS reactive B cells derived from the spleen of adult 'antigen-free' mice and from the spleen of neonatal conventional mice (Chapters 7 and 8). Both collections of hybridomas showed a preferential usage of the V_H gene families that are most proximal to the constant C_H region and showed a high frequency of multireactive (IgM) antibody-producing cells. This data suggests that the available B cell repertoire of adult 'antigen-free' mice resembles that of neonatal mice.

A summary of the data presented in this thesis is shown in Table 1. The relevance of the aforementioned results for the existing ideas about the selection of the different B cell repertoires will be discussed in more detail in the subsequent sections.

9.2. Development of B cell repertoires

In the mouse, the fetal liver is known as the organ where during early embryonic development the first cells from the B cell lineage, characterized by the surface antigen B220, are found at day 11 of gestation (Kincade, 1981; Velardi and Cooper, 1984). B lineage cells are transiently formed in the spleen during the burst of haemopoietic activity in this organ immediately before and after birth. The production of B lineage cells in the bone marrow is initiated relatively late in embryogenesis, around day 17, and continues throughout life. During the differentiation of B cells from uncommitted precursors the specificity repertoire of the B cells is being established.

The factors initiating the subsequent waves of generation of B cells are not clear. Interleukin 7, an interleukin produced by stromal cells, has recently been identified as an important growth factor for pre-B cells (Namen et al., 1988). Several well defined factors that promote growth and differentiation of myeloid precursors may also affect early B-lineage cells; these include IL-3, CSF-1 and GM-CSF (Paige et al., 1984; Palacios and Steinmetz, 1985). It has also been found that some of the IgM molecules which are being produced early in ontogeny can polyclonally stimulate the growth of other B cells (Kearney et al., 1989).

During fetal development the embryo is clearly not in direct contact with the exogenous antigenic environment and factors selecting this early B cell repertoire must be of endogenous origin. Allogeneic differences between mother and offspring influence the specificity repertoire, since the offspring normally seem to be tolerized for the non-inherited allogeneic differences (Claas et al., 1988). Exposure of the immune system to alloantigens during early fetal life is thought to be responsible for the induction of this unresponsiveness. Maternal Ig, especially those of the IgG class, are efficiently transferred to the embryo and might influence the specificity repertoire of the B cells through idiotypic-anti-idiotypic interactions (Bernabé et al., 1981). Since especially the IgG specificity repertoire of the mother reflects her antigenic experience, this might be a way of adapting the specificity repertoire of the fetus towards the antigenic environment it will encounter later in life. Obviously, other blood-borne antigens which are able to pass the blood-placenta barrier can function in a similar fashion. The influence of maternal IgG on the fetal development of the B cell repertoire is clearly decreased by breeding mice during many generations in the absence of exogenous antigenic stimulation. The GF-CD BALB/c colony was initiated by moving

Table 1 Comparison between adult GF-CD, conventional neonatal (CV-NEO) and conventional adult (CV-NI) BALB/c mice

	adult GF-CD	neonatal CV-NEO	adult CV-NI
Available repertoire			
Hybridoma production	LPS-stimulation	no LPS stimulation	LPS stimulation
V_H gene family usage	more PC7183	more PC7183; less J558	ad random[a]
Multireactivity	high frequency	high frequency	not tested
High connectivity	not tested	high frequency	low frequency[b]
Ly-1 B cells/spleen	6%	10%	1%[c]
Actual repertoire			
Number Ig-secr. cells			
IgM	100%	--	100%
IgG	<1%	--	100%
IgA	<1%	--	100%
Frequency Ag-specific Ig-secr. cells			
IgM	1 in 143	--	1 in 65
IgG	1 in 48	--	1 in 60,000
IgA	1 in 93	--	1 in 2,118
Serum Ig levels			
IgM	100%	--	100%
IgG	6%	--	100%
IgA	8%	--	100%
Ag-specific serum Ig levels to			
exogenous Ag	reduced	--	normal
endogenous Ag	normal	--	normal

Summary of data presented in this thesis except for:

a. Data from Dildrop et al., 1985; Holmberg et al., 1986a.

b. Data from Holmberg et al., 1986b.

c. Data from Hayakawa et al., 1985

pregnant GF BALB/c mice fed NI diet L-485 to another isolator whereafter they were fed the CD diet only. Their offspring, which had never directly contacted NI diet, but after weaning from maternal milk were changed to CD diet, were designated the first GF-CD generation (Pleasants et al., 1986). This first generation of GF-CD mice might have undergone the fetal selection of the specificity repertoire by maternal IgG, but since the colony is started off from already germfree mice, the antigenic experience of this generation of mothers was already severely reduced. In subsequent generations of GF-CD mice the maternal antigenic experience was minimized, but an influence of the exposure of the original BALB/c mice to exogenous antigens mediated by the maternal IgG through subsequent generations cannot completely be ruled out.

It is remarkable that in syngeneically mated GF-CD mice, which normally hardly produce any IgG (Chapters 4 and 5), the number of IgG-secreting cells (Chapter 6) and the serum IgG level (Chapter 5) are elevated during pregnancy. This might be caused by some male derived and other fetal antigens, or by hormonal changes. This IgG might be involved in the fetal selection of the B cell repertoire in GF-CD mice. Whether the specificity repertoire of the IgG antibodies induced by pregnancy is different from that of virgin GF-CD mice, which seems to be the same as the IgM specificity repertoire (Chapter 4), is unknown. The raise in IgG level seems to be temporary, since in old GF-CD mice which were earlier used for breeding purposes the serum IgG level was not different from young, virgin GF-CD mice (Chapter 5).

As mentioned earlier, a hugh wave of B cell production occurs in the spleen during the first 10 days after birth, while the liver progressively declines in B cell production. During this period conventional neonatal mice encounter the exogenous antigenic environment for the first time, which might shape the specificity repertoire of the formed B cell population. This production of B cells, however, is not predominantly caused by external antigenic stimulation, since in GF-CD mice also an exponential increase in the number of Ig secreting cells was found, although at a somewhat lower rate than in conventional mice (Chapter 3). During the exponential increase in B cell number a large number of activated B cells can be found in the neonatal spleen (Pereira et al., 1986). This is in contrast with the adult spleen, where a steady state in the number of B cells is established and only a limited number of activated B cells can be found (Pereira et al., 1986). Efficient fusing of B cells and a non-secreting myeloma fusion partner can only be established if the B cells are at the G_1-, G_2-, or S-phase of the cell cycle, because of the necessary synchronization of the two cell cycles of the fusion partners (Westerwoudt, 1986). This might explain why from neonatal spleen cells without additional stimulation many hybridomas can be established, while without additional stimulation attempts to establish hybridomas from adult splenic B cells are much less successful (unpublished observations; Holmberg et al., 1986a). In this

context it is difficult to discriminate between the available and actual neonatal repertoire, because many newly formed, 'available', neonatal B cells are activated and thus might participate in the actual repertoire. However, the majority of the high number (~ 50% of the nucleated cells) of large, activated B cells in the neonatal spleen are not actually secreting Ig and only a small, constant percentage (~ 1%) fully differentiate into Ig-secreting cells during the exponential expansion of the neonatal spleen. This population of large, activated B cells is probably the best fusion partner to produce Ig-secreting hybridomas. Therefore it might be better to define the repertoire as judged by hybridomas derived from neonatal spleen cells as the "activated available" repertoire (Chapters 7 and 8).

At the adult age a dynamic steady state in the number of B cells in the spleen is reached. A large proportion (~ 90%) of the splenic B cells are short-lived and the steady state is maintained by the continuous immigration of newly formed B cells from the bone marrow, which is at that time the most important production site of B cells (Freitas et al., 1986; Osmond, 1986). A small proportion of the splenic B cells are large, activated cells (~ 10%). Most large cells are cycling cells, involving cells secreting all different Ig classes (Pereira et al., 1986). These Ig-secreting cells constitute ~1% of the splenic B cells (Benner et al., 1982). Here a clear distinction can be made between the available and the actual repertoire, as represented by the newly formed and resting B cells and the Ig-secreting cells, respectively. The occurrence of large activated cells in the spleen is not dependent on exogenous antigenic stimulation, since comparable numbers were found in GF-CD mice and conventional mice (Pereira et al, 1986). The compartment of "actual" Ig-secreting cells is different in GF-CD and CV-NI mice. While the number of IgM-secreting cells does not differ between GF-CD and conventional mice, the numbers of IgG- and IgA-secreting cells are severely reduced in GF-CD mice (Pereira et al., 1986; Chapter 4).

9.3. Memory B cells

In the adult mouse a considerable proportion of the B cells can recirculate (Sprent, 1973). These B cells are memory B cells specific for and induced by previously experienced antigens. These cells occur not only in the peripheral blood, but also in all peripheral lymphoid organs like spleen, lymph nodes and mucosa associated lymphoid tissues in the intestine and the lung. These are the specialized sites where contact with exogenous antigens is most abundant and antigen-specific B and T cells are activated. Germinal centers are thought to be an important breeding site of memory cells (Wakefield et al., 1968; Kraal et al., 1982).

Memory B cells are ill-defined. In humoral immunity memory is defined as the

more rapid induction of antigen-specific Ig-secreting cells and increase of serum antibody titers. With respect to memory induction, antigens are usually divided into thymus-dependent (TD) and thymus-independent (TI) antigens (Mosier and Subbarao, 1981). T cells seem to play an important role in memory induction since in athymic nude mice, which have no functional T cells, memory to TD antigens can not be induced. Memory B cells are thought to be long-lived and memory to TD antigens can be transferred to X-irradiated hosts by long-living B cells if adequate T-cell help is provided (Feldbush, 1980; Koch et al., 1981). TI antigens usually do not induce memory B cells, although some of these antigens, mostly LPS-conjugates, can do so. For the latter TI-antigens it has been shown that the generation of memory B cells requires the continuous recruitment of newly formed bone marrow B cells (Colle et al., 1988; Burlen et al., 1988). The available B cell repertoire of memory B cells will obviously be influenced by exogenous antigenic stimulation, but it is difficult to study the available repertoire of the memory B cell population separately because it is not well possible to separate this population from the newly formed B cells.

9.4. Ly-1 B cells

A small subpopulation of B cells carries the, originally T cell related, Ly-1 marker on its cell surface. These Ly-1 B cells are thought to belong to a separate B cell lineage (reviewed in Herzenberg et al., 1986). This is based on experiments where reconstitution of irradiated mice with bone marrow cells resulted in the reconstitution of the "normal" B cells, but not of the Ly-1 B cells. Reconstitution with (IgM^+) B cells from the peritoneal cavity, which carry for 50% the Ly-1 marker, however, did give rise to Ly-1 B cells in the recipient. This was further substantiated by reconstitution experiments using congenic mice with different B cell markers. The number of Ly-1 B cells in the spleen changes during ontogeny. A relatively high fraction (10%) of neonatal splenic B lymphocytes carries the Ly-1 marker, while in the adult spleen of normal conventional mice only 1% of the B cells is Ly-1 positive.

In mice that are neonatally injected with allotype-congenic peritoneal cells, it is possible to obtain chimeric mice which possess, in addition to their own lymphocytes, a population of Ly-1 B cells of donor allotype (Förster and Rajewsky, 1987). Later in life the Ly-1 B cells appear to be the only donor-derived B cells in these mice. While they start off as a polyclonal population, they progressively become oligoclonal and even can become monoclonal (Förster et al., 1988; Tarlinton et al., 1988). Functionally the Ly-1 B cells appear to participate only in certain types of immune responses, mainly directed against bacterial antigens and self-antigens (Hayakawa et al., 1986; Casal et al., 1987; Förster and Rajewsky, 1987; Hardy et al., 1987). In addition they are

responsible for the production of a major fraction of the normal serum Ig (Herzenberg et al., 1986; Förster and Rajewsky, 1987). Recently it has been shown that a large proportion of the IgA-plasma cells in the gut of the mouse are probably derived from peritoneal Ly-1 B cells (Kroese et al., 1989).

In autoimmunity-prone mouse strains like NZB, MRL/Mp-lpr/lpr, BXSB and kd/kd mice the number of Ly-1 B cells is increased (Stall et al., 1988). When such mice are kept on a chronic energy intake restriction (CEIR) diet, not only their life span is expanded and the autoimmune phenomena are reduced, but also the number of Ly-1 B cells is reduced to proportions normally found in non autoimmunity prone strains (Ogura et al., 1989). The mechanism underlying the influence of the CEIR diet on the number of Ly-1 B cells is unclear. In the adult GF-CD mice which are fed the CD diet ad libitum a higher percentage and absolute number of Ly-1 B cells was found in the spleen compared to conventional controls. Autoimmune phenomena, however, were not observed and their life span was comparable to conventional syngeneic control mice (Pleasants et al., 1986). It therefore seems unlikely that the chemically defined diet directly influences on the Ly-1 B cell compartment.

9.5. The potential repertoire

The potential repertoire is determined by the number, structures and mechanisms of expression of the germline genes encoding antibodies plus the possible somatic variants derived from them. This repertoire can only be studied at the molecular level. The genetic elements and mechanisms responsible for the diversification of the Ig have been described in Chapter 1.

The germline array of V genes is an important source of antibody diversity in the immune system. The other diversification strategies include combinatorial and somatic mutation processes. Presumably, evolutionary selection has increased the number of V-region genes to make an extensive germline repertoire available for primary immune responses. The variable region of the heavy chain locus has been examined extensively in mouse and man. V_H gene families, defined by nucleotide sequence relationships, comprise distinct sets of highly related V_H genes (Brodeur and Riblet, 1984; Winter et al., 1985; Kofler, 1988; Reininger et al., 1988; Pennel et al., 1989b). While the different mouse V_H gene families are located in partially overlapping, discrete clusters (Blankenstein and Krawinkel, 1987; Rathbun et al., 1987; Brodeur et al., 1988), the human V_H gene families are located completely interspersed (Kodaira et al., 1986, Berman et al., 1988).

A premammalian divergence and at least some expansion of the major group

of V_H genes is suggested by the similarity between homologous pairs of V_H genes in mouse and man which is larger than between V_H genes of different families within either species (Rechavi et al., 1983). Recent examination of V_H gene copy number and polymorphism in different mouse IgH-V haplotypes showed that after this initial expansion of the locus only small duplications and deletions occurred, each involving one or at most a few members of a single V_H gene family, presumably allowing for the maintenance of at least a minimum V-region pool size (Tutter and Riblet, 1988). The difference in interspersion between mouse and man suggests that unequal recombination between homologous genes may frequently occur within this locus. In this respect it is interesting that the most interspersed V_H gene families in the mouse are the most 3' families Q52 and PC7183, which are also involved in early rearrangements during ontogeny (see section 9.6.). It might be that the local chromatin structure facilitates both rearrangement and recombination in this area.

The variable region of the mouse κ light chain locus is less well defined than the heavy chain locus. Based on amino acid similarity up to the invariant tryptophan in position 35, the 100-300 estimated mouse V_κ light chain genes have been classified in 24 V_κ groups (Potter et al., 1982). However, whether these V_κ groups correspond to non-overlapping V_κ gene families (analogous to V_H gene families) is not clear. Recent studies, using DNA probes corresponding to the different protein groups, showed that several V_κ gene families defined by these probes overlap. This suggests that the V_κ gene complex, in contrast to the V_H gene complex, is more a continuum of related sequences (D'Hoostelaere et al., 1988; Kofler et al., 1989). The λ light chain locus has the smallest variable region part and consequently contributes only to a small extent to the diversification of the antibody repertoire. It consists only of two V genes and three distinct J-C complexes (Selsing et al., 1989). In the mouse this is associated with a limited expression of the λ light chain (5%) in the serum Ig. Next to the limited heterogeneity of the λ gene locus, the sequential rearrangement of the different V genes that is observed during B cell differentiation might also be involved in the difference in κ and λ light chain expression. At first the heavy chain locus is rearranged and subsequently the κ and λ light chain loci are rearranged. When a functional Ig molecule is formed, this shuts down further recombination, giving the λ locus less chance to rearrange (Storb et al., 1986).

Recently two genes have been cloned that are differentially expressed in pre-B cells. A gene termed λ5, because of significant homology to Cλ and Jλ sequences and $V_{pre\text{-}B}1$, owing its name to its homology to IgV genes (Kudo et al., 1987; Kudo and Melchers, 1987). These genes, which are closely linked, are expressed without rearrangement in neoplastic pre-B cell lines. It is thought that the $V_{pre\text{-}B}1$ gene product may be part of the μ-λ5 complex found on the surface of pre-B cell lines (Pillai and

Baltimore, 1987; Hendershot et al., 1988). This might be the signal that the pre-B cell has undergone a successful μ heavy-chain gene rearrangement and should begin the light chain rearrangement process. On the other hand, this product could be the target for the earliest selection of the expressed V_H germline repertoire (Forni et al., 1979).

9.6. The available repertoire

The available repertoire can be defined as the set of diverse antibody molecules that are expressed by immunocompetent but resting B cells. These include both newly-formed and memory B cells. The available repertoire can be studied by determining the number of B cells expressing different antigen specificities and by determining the V genes that are being expressed in those B cell populations. The antigen specificity repertoire has been studied by determining the frequencies of antigen-specific B cell clones with different methods. The in vitro splenic focus assay (Klinman and Aschinazi, 1971), the in vitro limiting dilution assay (Lefkovits, 1979), long term bone marrow culture (Whitlock et al., 1984), semi-solid agar cloning (Paige et al., 1984) and production of Ig-secreting hybridomas (Köhler and Milstein, 1975) are being used for analysis of antibody specificities among different B cell populations. Furthermore, the ability of the available B cells to give rise to an immune response to different antigens after immunization is also a measure of the capacities of the available repertoire.
The V genes expressed in the available repertoire have been examined by RNA-analysis of A-MuLV transformed pre-B cell lines (Yancopoulos et al., 1984), B cell hybridomas (Perlmutter et al., 1985), in vitro cultured colony forming (pre-)B cells (Wu and Paige, 1986; Schulze and Kelsoe, 1987) and by direct in situ hybridization (Jeong et al., 1988). Furthermore, V gene expression was analyzed by quantitative analysis of total splenic RNA (Yancopoulos et al., 1988) and production of a phage library with expressed V genes from total splenic RNA (Sheenan and Brodeur, 1989).

Immunization of adult GF-CD mice with fibrin resulted in a good secondary type response as shown by the isolation of hybridomas which produce antibodies with high affinity to fibrin (Gargan et al., 1988). Also for some other antigens it was possible to produce monoclonal antibodies from GF-CD mice, while the production of such monoclonal antibodies was unsuccessful in conventional and even in germfree mice (Gargan, personal communication). This shows that the available repertoire of GF-CD mice is fully competent to respond to exogenous antigens. It might be speculated that the absence of competition between different antigens in GF-CD mice is responsible for the success in monoclonal antibody production from those mice.

For the estimation of precursor frequencies it is often necessary to activate the resting B cells, either polyclonally or antigen-specifically, into an effector compartment,

which then can be analyzed for antigen specificities and V gene usage. Most in vitro culture systems have the advantage of screening large B cell populations at the clonal level, but the individual B cell clones can only be cultured for a limited time and in limited numbers. On the other hand, the immortalization of B cells by A-MuLV transformation or by production of hybridomas allows an in depth analysis of individual B cells.

During ontogeny the available repertoire is established for the first time. It has been found that certain immune responses appear in an ordered sequence during ontogeny. B cells specific for polysaccharide antigens such as phosphoryl choline (PC) (Fung and Köhler, 1980), α 1→3 dextran (Stohrer and Kearney, 1984) and inulin (Bona et al., 1979) did not become functional until or after the first week of life, while certain DNP- and TNP-specific clonotypes consistently appear before birth (Klinman and Press, 1975). Also the splenic repertoire against the hemagglutinin molecule of influenza virus at 12-14 days of age is more restricted than at the adult age (Cancro et al., 1979). This programmed development of the specificity repertoire seems to occur independent of exogenous antigenic stimulation, since in germfree mice the same pattern was found (Sigal et al., 1976). During this early development the B cell repertoire is highly susceptible to tolerizing and modulating influences. Idiotypic network interactions might play an important role during the establishment of the early repertoire. Experiments of Vakil and Kearney have shown that injection of minor amounts of anti-idiotypic antibodies at certain time points during ontogeny clearly influence the repertoire of the corresponding B cells and that this perturbation is long lasting (Vakil and Kearney, 1986; Vakil et al., 1986b). The early B cell repertoire has been investigated in hybridomas from fetal liver and neonatal spleen (Holmberg et al., 1984; Vakil and Kearney, 1986; Glotz et al., 1988). From these organs hybridomas can easily be established, because many activated B cells are found in these organs, even when exogenous antigenic stimulation is avoided (Pereira et al., 1986). Among such hybridomas a high proportion was found which produce antibodies which can react with multiple antigens from both exogenous and endogenous origin (Chapter 8). These multireactive antibodies include the anti-idiotypic antibodies mentioned earlier which can influence the available specificity repertoire and this might be one of their functions. Another possible function of these multireactive antibodies may be a first line of defense which can react with a broad spectrum of antigenic structures.

At the adult age the available repertoire represents a dynamic steady state of newly formed B cells, imported from the bone marrow, and memory B cells. At present little is known about the selective forces which determine which newly formed B cells are allowed to enter the periphery and even less is known how B cells become memory B cells after the first contact with antigens. The production rate of precursor

B cells in the bone marrow greatly exceeds the number of B cells migrating to the periphery (Opstelten and Osmond, 1983). This transition could be prone to selection mechanisms. When exogenous antigenic stimulation is avoided, the splenic available repertoire as determined in hybridoma collections derived from LPS-reactive B cells of adult GF-CD mice resembles that derived from 'naturally activated' neonatal spleen cells. Both collections show a high frequency of hybridomas producing multireactive antibodies (Chapter 8). Such hybridomas are less frequently found in hybridoma collections derived from LPS-reactive B cells from adult conventional mice (Holmberg et al., 1986b). This suggests that exogenous antigenic stimulation influences the establishment of the adult 'available' B cell specificity repertoire. It remains to be established whether this selection is performed directly by exogenous antigens or by idiotype-anti-idiotype interactions which reflect the interactions of peripheral B cells with the exogenous antigens. Experiments employing limiting dilution analysis of LPS-reactive B cells did not show differences in frequency of antigen-specific precursors between GF-CD and CV-NI mice (Hooijkaas et al., 1985). Furthermore the T15-idiotype dominance in responses of BALB/c mice to phosphorylcholine is similar in GF-CD and CV-NI mice (Etlinger et al., 1986). Also the frequency of phosphorylcholine specific B cells determined by the splenic focus assay was the same in germfree and conventional mice (Sigal et al., 1975). All these experiments, however, tested only one antigen at the time and did not measure multireactivity. Direct comparison between the frequencies of multireactive LPS-activated B cells in adult GF-CD and CV-NI mice still needs to be done.

The variable region genes that are being expressed in the available repertoire have been investigated mostly for the heavy chain genes, amongst others during B cell development. Several studies with different approaches have demonstrated that V_H gene usage is biased towards C_H proximal V_H gene segments in cell lines derived from fetal or newborn mice, while V_H gene usage is more normalized - that is, more related to family size instead of family position - in hybridomas, spleen cultures and LPS-stimulated splenocytes from adult spleen (Yancopoulos et al., 1984; Dildrop et al., 1985; Perlmutter et al., 1985; Holmberg et al., 1986a; Wu and Paige, 1986; Schulze and Kelsoe, 1987; Jeong et al., 1988). This 'normalization' does not fully occur under antigen-free conditions, pointing to an influence of exogenous antigens on this process (Chapter 7). The position dependent V_H gene family usage correlated with the occurrence of the above mentioned multireactive antibodies (Holmberg, 1987; Chapter 8).

The contribution of the Ly-1 B cell lineage to the repertoire found in hybridomas derived from neonatal spleen and LPS-reactive B cells of adult GF-CD mice is not clear. The Ly-1 B cell population has been shown to produce autoreactive antibodies

(Hayakawa et al., 1986; Herzenberg et al., 1986) and both in neonatal spleen (10%) and in the spleen of adult GF-CD mice (6%) a higher percentage of Ly-1 B cells occur than the 1% that is usually found in the spleen of conventional adult mice (Hayakawa et al., 1983; Chapter 6). The percentage of hybridomas originating from GF-CD mice and producing multireactive antibodies (~ 50%; Chapter 8), however, cannot completely be explained by Ly-1 B cells, unless a preferential fusing of Ly-1 B cells is supposed. Unfortunately hybridomas do not express the Ly-1 marker, therefore this possibility cannot directly be investigated. The expression of Leu-1 RNA (the human analog of Ly-1) in Leu-1$^-$ B cell lines derived from Leu-1$^+$ B cells which has been shown for some human B cell clones, might give better insight in this unresolved question (Schutte, personal communication). The V_H gene family usage in purified Ly-1 B cells derived from the peritoneum of adult mice shows a preferential usage of V_H11, but this was not found in neonatal tissues with relatively high percentages of Ly-1 B cells (Andrade et al., 1989). The preferential usage of V_H11, which encodes antibodies specific for bromelain treated mouse red blood cells, is thought to be due to antigenic selection (Lalor et al., 1989; Pennel et al., 1989a). In older animals a restricted V_H gene usage has been found in Ly-1 B cells (Förster et al., 1988; Tarlinton et al., 1988). These data suggest that Ly-1 B cells start from expressing a broad V_H repertoire, but, in the absence of significant turnover from uncommitted precursors, are submitted to strong selective forces during life.

It has also been suggested that the biased usage of V_H gene families could account for the developmental pattern of different antigen specificities occurring during ontogeny (Yancopoulos et al., 1984; Perlmutter, 1987). Some correlation could be shown in the response of mice to influenza hemagglutinin (HA). Genes from most V_H gene families can code for HA-specific antibodies, but clonotypes prevalent during the first week of life disproportionately use PC7183, while hybridomas representative of the repertoire in two-week-old individuals preferentially use S107, 3660 and X24 (Kienker et al., 1988).

A biased repertoire for the 3' V_H gene families could also be shown in pre-B cells from adult bone marrow although not as striking as in fetal liver and neonatal spleen (Yancopoulos et al., 1984; Denis et al., 1989), suggesting that the normalization in adult mice occurs during the differentiation of uncommitted precursors into newly formed B cells. At which stages of B cell differentiation and by which factors this selection takes place, is still unknown (Freitas et al., 1988).

9.7. The actual repertoire

The actual specificity repertoire can be defined by the set of Ig molecules that are actually secreted by Ig-secreting cells. It is also represented by the serum Ig molecules. The actual repertoire can be investigated by direct measurement of the frequency of antigen-specific Ig-secreting cells and the measurement of antigen-specific serum Ig levels. The role of antigenic selection on the actual repertoire is obvious. According to the clonal selection theory (Burnet, 1957) antigens activate the appropriate B cell clones from the available repertoire, leading to proliferation and differentiation of clones of antigen-specific Ig-secreting cells. This can be shown after immunization with antigen by detection of specific antibody production and antigen specific Ig-secreting cells. In not intentionally immunized mice the 'background' Ig-secreting cells and serum Ig represent the actual specificity repertoire (Benner et al., 1982). A clear influence of age and immune status on the background Ig production has been shown (Benner et al., 1981). Even under antigen-free conditions many Ig-secreting cells and serum Ig can be detected (Hooijkaas et al., 1984; Chapters 3-6). Normal numbers of IgM-secreting cells and normal serum IgM levels are found in GF-CD mice, while the numbers of IgG- and IgA-secreting cells and the serum IgG and IgA levels are severely reduced (Chapters 3-5). Frequencies of antigen-specific IgG- and IgA-secreting cells, but not of IgM-secreting cells, are also different between GF-CD and conventional mice (Chapter 4). This is probably caused by the selection of IgG- and IgA-secreting cells by exogenous antigens during normal immune responses in conventional mice. In GF-CD mice the specificity repertoires of the (few) IgG- and IgA-secreting cells resemble that of the IgM-secreting cells (Chapter 4), suggesting ad random switching from IgM to the other isotypes in those mice.

It can be hypothesized that the actual repertoire can be subdivided into an antigen-dependent and an antigen-independent compartment. The antigen-dependent compartment consists of B cells which, upon activation by antigen, give rise to primary and secondary humoral immune responses with antigen specific antibody production, memory induction, isotype switch and affinity maturation. The function of the antigen-independent compartment is still speculative. It is established early in ontogeny (Chapter 3) and consists of IgM-secreting cells. This compartment may be involved in the establishment and maintenance of an idiotypic network (Kearney and Vakil, 1986). Probably this compartment also contains the multireactive antibody-producing cells (Klinman et al., 1988). They contribute to a large extent to the serum IgM pool (Chapter 5). In serum of normal individuals such multireactive (including autoreactive) antibodies can easily be detected (Guilbert et al., 1982). Recently it has been shown that serum levels of such "natural antibodies" with anti-idiotypic connectivity show a

very regular pattern of occurrence compared to each other, suggesting a regulated dynamic steady state (Lundkvist et al., 1989). It has also been shown that by injection of natural anti-idiotypic antibodies the (auto-immune) response to the acetylcholine receptor could be prevented (Sundblad et al., 1989). This suggests a role for these antibodies in preventing autoimmune diseases. The therapeutic use of pooled Ig to suppress autoantibodies to factor VIII is probably based on such antibodies (Rossi et al., 1989).

The relationship between the antigen-dependent and antigen-independent B cell compartments is unclear. Are they recruted from the same pool of 'available' newly formed B cells or not? Which internal factors (idiotypes?) drive the 'antigen-independent' B cells into the actual compartment? Considering their stability and occurrence even under antigen-free conditions, they must play an important role in the normal physiology of the immune system. For a better understanding of this important B cell population further experimentation is needed.

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SUMMARY

The antibody specificity repertoire can be defined as the total number of different antibodies that the immune system is able to produce. Since every clone of B cells produces only one type of antibody, the antibody specificity repertoire can be regarded to be the same as the B cell repertoire. The B cell repertoire can be subdivided into three categories. The *potential* repertoire is determined by the number, structure and mechanisms of expression of the germline genes encoding immunoglobulin (Ig) molecules. The *available* repertoire is defined as the set of diverse antibody molecules that are expressed by immunocompetent but resting B cells. The *actual* repertoire is represented by the set of antibodies that is actually secreted by B cells. Little is known about the factors selecting the available repertoire from the potential repertoire and the actual repertoire from the available repertoire.

According to the clonal selection theory antigens select B cells from the available repertoire with appropriate antigen receptors into the actual repertoire. Less is known about the influence of antigenic stimulation on the establishment of the available repertoire. The influence of exogenous antigenic stimulation on the establishment of the B cell repertoire is difficult to study, since the organism is surrounded by exogenous antigens from birth on. The purpose of the studies presented in this thesis was to obtain more information concerning the influence of exogenous antigenic stimulation on the establishment of the B cell repertoire. This was done by using neonatal and adult germfree BALB/c mice fed a chemically defined ultrafiltered 'antigen-free' diet (GF-CD mice) and conventional syngeneic control mice fed natural ingredient diet (CV-NI mice).

The development of the compartment of Ig-secreting cells was determined in neonatal GF-CD and CV-NI mice from birth to the young adult age. During the first few weeks of life the total number of Ig-secreting cells in the spleen developed faster in CV-NI mice than in GF-CD mice. At the young adult age equal numbers of IgM-secreting cells were found in both groups of mice, but the numbers of IgG- and IgA-secreting cells were reduced in GF-CD mice. The frequencies of antigen-specific IgM-secreting cells were the same in both groups of mice during the observation period. This suggests that the ontogenetic appearance of Ig-secreting cells in the spleen and the specificity repertoire of the IgM-secreting cells are independent of exogenous antigenic stimulation. However, after birth the rate of development of the Ig secreting cell compartment was enhanced by environmental antigenic stimulation (Chapters 3 and 4).

At the adult age the actual repertoire was studied by determination of the total numbers IgM-, IgG- and IgA-secreting cells and the frequencies of antigen-specific

IgM-, IgG- and IgA-secreting cells in the spleen, bone marrow and lymph nodes of GF-CD and CV-NI mice. The numbers of IgM-secreting cells were the same in adult GF-CD and CV-NI mice, while the numbers of IgG- and IgA-secreting cells were severely reduced in GF-CD mice. Frequencies of antigen-specific IgG- and IgA-secreting cells, but not of IgM-secreting cells, were also different between GF-CD and conventional mice. This suggests that the actual IgM specificity repertoire is established independent of exogenous antigenic stimulation, while the actual IgG and IgA specificity repertoires are highly influenced by exogenous antigens (Chapter 4).

This is also reflected in the serum. The serum concentration of IgM was comparable between GF-CD and CV-NI mice, but the serum levels of IgG and IgA were severely reduced in GF-CD mice. Among the IgM antibodies a comparable titer of antibodies specific for the endogenously occurring polysaccharide antigen 3-fucosyllamine was found in GF-CD and CV-NI mice. On the other hand, the titers to some exogenous polysaccharide antigens were very low in GF-CD mice compared to conventional controls (Chapter 5).

In pregnant GF-CD mice a temporary rise in the number of IgG- and IgA-secreting cells was found. This suggests that endogenous factors (e.g. hormones or fetal antigens) can also influence the actual IgG and IgA production (Chapter 6).

The available repertoire was studied by the determination of the usage of V_H gene families in a collection of hybridomas of LPS-reactive B cells derived from the spleen of adult GF-CD mice and a hybridoma collection from neonatal splenic B cells. Both collections of hybridomas showed a preferential usage of the V_H gene family that is most proximal to the constant C_H region (Chapter 7).

Both collections were screened against a large panel of exogenous and endogenous antigens and were shown to have a high frequency of multireactive antibody-producing cells. This data suggests that the available repertoire of adult GF-CD mice resembles that of neonatal conventional mice (Chapter 8).

The preceeding data are discussed in Chapter 9. In conclusion, the results described in this thesis show that early in ontogeny the B cell repertoire consists of a large number of multireactive IgM-producing B cells, which show a preferential usage of the most C_H-proximal V_H gene families. If exogenous antigenic stimulation is avoided the available B cell repertoire of adult mice resembles that of neonatal mice. In the actual B cell repertoire a distinction can be made between an antigen-independent compartment of mainly IgM-secreting cells and an antigen-dependent compartment consisting mainly of IgG- and IgA-secreting cells. Most probably the antigen-dependent compartment is responsible for the normal immune responses against exogenous antigens, while the antigen-independent compartment plays an important role in the homeostasis and regulation of the immune system by idiotypic network interactions.

SAMENVATTING

Het antilichaam-specificiteitsrepertoire kan worden gedefinieerd als het totaal aantal verschillende antilichamen dat het immuunsysteem kan produceren. Omdat elke kloon van B cellen slechts één uniek type antilichaam maakt, kan het antilichaam specificiteitsrepertoire beschouwd worden als het B cel repertoire. Het B cel repertoire kan worden onderverdeeld in drie categorieën. Het *potentiële* repertoire wordt bepaald door het aantal, de struktuur en de expressiemechanismen van de genen die coderen voor de immunoglobuline (Ig) moleculen. Het *beschikbare* repertoire is gedefinieerd als die antilichaam specificiteiten die door immunocompetente maar rustende B cellen tot expressie worden gebracht. Het *feitelijke* repertoire wordt vertegenwoordigd door de antilichamen die door B cellen worden uitgescheiden. Er is weinig bekend over de factoren die het beschikbare repertoire selecteren uit het potentiële repertoire en het feitelijke repertoire uit het beschikbare repertoire.

Volgens de klonale selektietheorie recruteren antigenen de B cellen van het beschikbare repertoire die een geschikte antigeenreceptor hebben, in het feitelijke repertoire. Veel minder is bekend over de invloed van antigene stimulatie op het tot stand komen van het beschikbare repertoire. De invloed van exogene antigene stimulatie op de vorming van het B cel repertoire is moeilijk te bestuderen, omdat het organisme vanaf de geboorte is omgeven door exogene antigenen. Het doel van de studies die in dit proefschrift worden beschreven, was om meer informatie te verkrijgen over de invloed van exogene antigene stimulatie op het ontstaan van het B cel repertoire. Dit is gedaan door gebruik te maken van neonatale en volwassen BALB/c muizen die gehouden werden onder kiemvrije omstandigheden en gevoed werden met een 'antigeen-vrije', gefilterde oplossing van chemisch gedefinieerde voedingsstoffen (GF-CD muizen). Ter vergelijking werden conventionele syngene controle muizen gebruikt die een normaal dieet hadden gekregen (CV-NI muizen).

De ontwikkeling van het compartiment van Ig-secernerende cellen werd bepaald in neonatale GF-CD en CV-NI muizen vanaf de geboorte tot de jong volwassen leeftijd. Gedurende de eerste weken ontwikkelde het totaal aantal Ig-secernerende cellen in de milt zich sneller in CV-NI muizen dan in GF-CD muizen. In jong volwassen muizen werden vergelijkbare aantallen IgM-secernerende cellen gevonden in beide groepen, maar de aantallen IgG- en IgA-secernerende cellen waren verlaagd in GF-CD muizen. De frequentie van antigeen-specifieke IgM-secernerende cellen was gedurende de observatieperiode hetzelfde in beide groepen muizen. Dit suggereert dat de verschijning van Ig-secernerende cellen in de milt tijdens de ontogenie en het specificiteitsrepertoire van de IgM-secernerende cellen onafhankelijk zijn van exogene antigene stimulatie. De snelheid van de ontwikkeling van het compartiment Ig-

secernerende cellen wordt echter versneld door antigene stimulatie vanuit de omgeving (Hoofdstuk 3 en 4).

Op de volwassen leeftijd werd het feitelijke repertoire bestudeerd door bepaling van het totaal aantal IgM-, IgG- en IgA-secernerende cellen en de frequentie van antigeen-specifieke IgM-, IgG- en IgA-secernerende cellen in de milt, het beenmerg en de lymfeklieren van GF-CD en CV-NI muizen. Het aantal IgM-secernerende cellen was hetzelfde in volwassen GF-CD en CV-NI muizen, terwijl het aantal IgG- en IgA-secernerende cellen aanzienlijk was verlaagd in GF-CD muizen. De frequenties van antigeen-specifieke IgG- en IgA-secernerende cellen, maar niet van IgM-secernerende cellen, waren verschillend in GF-CD en conventionele muizen. Dit suggereert dat het feitelijke IgM specificiteitsrepertoire onafhankelijk van exogene antigene stimulatie tot stand komt, terwijl het feitelijke IgG en IgA specificiteitsrepertoire sterk wordt beïnvloed door exogene antigenen (Hoofdstuk 4).

Hetzelfde werd gevonden in het serum, aangezien de serumconcentratie van IgM vergelijkbaar bleek te zijn bij GF-CD en CV-NI muizen, maar de serumniveaus van IgG en IgA sterk verlaagd waren in GF-CD muizen. Voor wat betreft de IgM antilichamen werd een vergelijkbare titer van antilichamen gevonden die specifiek zijn voor het endogeen voorkomende polysaccharide antigeen 3-fucosyllamine in GF-CD en CV-NI muizen, terwijl de titers tegen enkele exogene polysaccharide antigenen sterk verlaagd waren in GF-CD muizen t.o.v. conventionele controle muizen (Hoofdstuk 5).

In zwangere GF-CD muizen werd een tijdelijke verhoging van het aantal IgG- en IgA-secernerende cellen gevonden. Dit suggereert dat endogene factoren (bijvoorbeeld hormonen of foetale antigenen) ook van invloed kunnen zijn op de feitelijke IgG en IgA produktie (Hoofdstuk 6).

Het beschikbare repertoire werd bestudeerd door het bepalen van het gebruik van de V_H genfamilies in een collectie hybridomen die gemaakt was van LPS-reactieve B cellen afkomstig uit de milt van volwassen GF-CD muizen en een collectie hybridomen van B cellen uit de milt van neonatale conventionele muizen. Beide collecties hybridomen vertoonden een preferentieel gebruik van de V_H genfamilie die het dichtst is gelegen bij de genen die coderen voor het constante deel van de Ig zware keten (Hoofdstuk 7).

Beide collecties werden getest op het vermogen van de geproduceerde antilichamen om een groot aantal verschillende exogene en endogene antigenen te binden. Daarbij bleek dat een relatief groot deel van de hybridomen in beide collecties multireactieve antilichamen produceerde. Deze gegevens suggereren dat het beschikbare specificiteitsrepertoire van volwassen GF-CD muizen vergelijkbaar is met dat van neonatale muizen (Hoofdstuk 8).

Bovenstaande gegevens worden bediscussieerd in Hoofdstuk 9. Concluderend

laten de resultaten die beschreven zijn in dit proefschrift zien, dat vroeg in de ontogenie het B cel repertoire een groot aantal multireactieve B cellen omvat die preferentieel gebruik maken van de V_H gen familie die het dichtst bij de C_H genen ligt. Wanneer exogene antigene stimulatie wordt vermeden, lijkt het beschikbare repertoire van volwassen muizen op dat van neonatale muizen. In het feitelijke B cel repertoire kan onderscheid worden gemaakt tussen een antigeen-onafhankelijk compartiment van voornamelijk IgM-secernerende cellen en een antigeen-afhankelijk compartiment van voornamelijk IgG- en IgA-secernerende cellen. Hoogst waarschijnlijk is het antigeen-afhankelijke compartiment verantwoordelijk voor de normale immuunrespons tegen exogene antigenen, terwijl het antigeen-onafhankelijke compartiment een belangrijke rol speelt in de homeostase en regulatie van het immuunsysteem via idiotype-netwerk interacties.

List of abbreviations

BSA	Bovine serum albumin
APC	antigen-presenting cell(s)
B-CLL	B cell chronic lymphocytic leukemia
BM	Bone marrow
CDR	Complementarity-determining region
CEIR	Chronic energy-intake restriction
C_H	Constant part of Ig heavy chain
C_L	Constant part of Ig light chain
CV-NI	Conventional mice fed natural ingredient diet
CV-NEO	Conventional neonatal mice
DNP	Dinitrophenyl
E-PFC	ELISA-plaque-forming cell
3-FL	3-Fucosyllactosamine
GF-CD	Germfree mice fed a chemically defined ultrafiltered diet
HA	Hemagglutinin
Ig	Immunoglobulin
J_H	Joining region of the heavy chain locus
J_L	Joining region of the light chain locus
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
NIP	5-iodo-3-nitrophenyl
NNP	4-hydroxy-3,5-dinitrophenyl
PC	Phosphorylcholine
PFC	Plaque-forming cell
PLN	Peripheral lymph nodes
PPC	Peptidoglycan-polysaccharide complexes
SCID	Severe combined immunodeficiency
SN	Supernatant
SPF	Specific pathogen-free
SRBC	Sheep red blood cells
TD	Thymus dependent
TI	Thymus independent
TNP	2,4,6-trinitrophenyl
V_H	Variable part of Ig heavy chain
V_L	Variable part of Ig light chain

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