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HET B CEL REPERTOIRE VAN DE MUIS

ANTILICHAAMSPECIFICITEITEN EN IMMUNOGLOBULINE (SUB) KLASSEN VERDELING

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. M.W. VAN HOF
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 15 MEI 1985 TE 15.45 UUR

Door

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geboren te Utrecht

1985
OFFSETDRUKKERIJ KANTERS B.V.,
ALBLASSERDAM

Begeleidingscommissie

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Dit proefschrift werd bewerkt binnen de vakgroep Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam.

Het onderzoek werd mede mogelijk gemaakt door financiële steun van de Nederlandse Stichting voor Medisch Wetenschappelijk Onderzoek (FUNGO) en het Interuniversitair Instituut voor Radiopathologie en Stralenbescherming (IRS).

Aan Titia

Aan mijn ouders

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VOORWOORD

Gewervelde dieren bezitten een aantal middelen om in het lichaam binnengedrongen vreemde elementen zoals bacteriën, virussen en schimmels onschadelijk te maken. Naast een systeem dat bestaat uit fagocyten ('eetcellen'), waaronder granulocyten en macrofagen, die *aspecifiek* bijvoorbeeld bacteriën kunnen opeten, hebben zij de beschikking over een afweersysteem dat uit lymfocyten bestaat: het *immuunsysteem*. Dit zorgt voor een *specifieke* afweer en reageert bovendien bij een tweede contact met een vreemd element (ook antigeen genoemd) heftiger dan de eerste keer, wat erop duidt dat het een *geheugen* bezit. *Specificiteit* en *geheugen* vormen de hoofdkenmerken van het immuunsysteem. De lymfocyten, behorend tot de witte bloedcellen of leukocyten, kunnen in twee groepen verdeeld worden: T lymfocyten, die zorgen voor de zogenaamde cellulaire immuniteit, en B lymfocyten, die zorgen voor de antilichaamvorming, de zogenaamde humorale immuniteit. Een voorbeeld van cellulaire immuniteit is de immuunreactie van T lymfocyten tegen door virus geïnfecteerde cellen. Zogenaamde cytotoxische T cellen vernietigen zulke geïnfecteerde cellen. T cellen vervullen vaak een belangrijke rol bij de regulatie van immuunreacties. T helper cellen zijn meestal nodig om B cellen te stimuleren tot antilichaamvorming. Andere typen T cellen kunnen de cellulaire en humorale immuunreacties weer remmen. B lymfocyten kunnen uitgroeien tot klonen plasmacellen die antilichamen produceren. Scheikundig gezien behoren antilichamen tot een groep eiwitten, die ook wel immunoglobulinen worden genoemd. Deze immunoglobulinen spelen een belangrijke rol bij de afweer tegen bacteriën en virussen.

In feite is het immuunsysteem in staat om tegen ieder, in de natuur voorkomend, antigeen antilichamen te maken. Sterker nog, ook tegen door de chemische industrie geproduceerde verbindingen die tot dan toe niet bekend waren, kan het reageren. Men neemt daarom aan dat het totale repertoire aan antilichaam-specificiteiten compleet is, dat wil zeggen, tegen elk denkbaar antigeen kan reageren, ook tegen antigenen die deel uitmaken van het eigen lichaam. Dat dit laatste gewoonlijk niet gebeurt, wordt toegeschreven aan het feit dat het immuunsysteem in staat is om agressieve reakties tegen zichzelf te vermijden, al is niet precies bekend op welke wijze. Hoe het immuunsysteem het klaar speelt om zo'n grote diversiteit aan antilichamen te produceren, is lange tijd onduidelijk geweest en ook nu nog zijn er vele vragen omtrent regulatiemechanismen onbeantwoord.

Binnen het immuunsysteem vindt een voortdurend proces van afbraak en vernieuwing plaats. Per seconde worden er in de mens ongeveer 1 miljoen lymfocyten en 10 biljoen immunoglobuline-moleculen gevormd. Aanvankelijk werd gedacht dat antigenen het immuunsysteem instrueerden om een passend antilichaam te maken. Met deze *instructietheorie* was gemakkelijk te verklaren waarom het immuunsysteem in staat is om specifiek te reageren tegen elk mogelijk antigeen. Maar omdat kon worden aangetoond dat de specificiteit van een antilichaam bepaald wordt door zijn aminozuursamenstelling en dus door het DNA (deoxyribonucleinezuur), de drager van erfelijke eigenschappen in de celkern, werd deze theorie verlaten en een nieuwe opgesteld: de *klonale selectietheorie*. Deze is inmiddels uitgegroeid tot het centrale dogma binnen de immunologie en gaat uit van twee principes. Het eerste is dat een lymfocyt antilichamen produceert die allemaal dezelfde specificiteit bezitten en dus allemaal tegen hetzelfde antigeen kunnen reageren. Het tweede principe is dat alle dochtercellen van een lymfocyt, een 'kloon' lymfocyten, eveneens

alle antilichamen produceren van dezelfde specificiteit. Volgens de *klonale selectietheorie* bestaat het immuunsysteem uit een groot aantal klonen van lymfocyten die alle voorbestemd zijn om antilichamen met een eigen specificiteit te maken. Iedere lymfocyt in een kloon heeft receptoren op zijn celoppervlak met precies dezelfde specificiteit als de antilichamen die gemaakt zullen worden na antigene stimulatie. Door middel van deze receptoren *selecteren* de antigenen die lymfocyten, die een passende specificiteit hebben. Aangezien iedere kloon lymfocyten zijn eigen specifieke antilichamen maakt, kan het aantal verschillende antilichamen nooit groter zijn dan het aantal verschillende klonen. Het specificiteitsrepertoire bij de mens wordt geschat op 10 tot 100 miljoen verschillende antilichamen, maar het is inmiddels duidelijk geworden dat slechts een paar honderd genen verantwoordelijk zijn voor de productie van deze diversiteit. Dit is mogelijk doordat er tijdens de productie van lymfocyten herschikkingen en mutaties optreden in dat deel van het DNA dat codeert voor het antigeenbindende deel van de immunoglobulinen.

Gebleken is dat antilichamen niet alleen reageren tegen vreemde antigenen, maar ook tegen elkaar, aangezien het deel van het antilichaammolecuul dat het antigeen bindt ook zelf als antigeen fungeert. Hierdoor worden dus anti-antilichamen opgewekt waartegen opnieuw B lymfocyten kunnen reageren, enz., tot een bepaald evenwicht is bereikt. Deze waarnemingen hebben geleid tot de *netwerktheorie* waarin het immuunsysteem wordt beschouwd als een netwerk van miljoenen elkaar beïnvloedende elementen. Elk element bestaat uit een kloon lymfocyten en de door hen geproduceerde antilichamen, met elk een eigen specificiteit, die elkaar geheel of gedeeltelijk complementeren. De *netwerktheorie* heeft grote invloed op het huidige immunologische denken.

Tot dusver is er nog weinig bekend over de mechanismen die een rol spelen bij het ontstaan van het antilichaam-specificiteitsrepertoire. Is dit een autonoom proces dat volgens bepaalde regels verloopt, of zijn er regulerende invloeden van buitenaf? Wij besloten om hiernaar onderzoek te doen bij de muis, omdat voor dit proefdier een aantal geschikte testsystemen voorhanden is. Miljoenen B lymfocyten worden per dag gevormd en de vraag is of zij in staat zijn ook bij het ouder worden van het individu het antilichaam-specificiteitsrepertoire op peil te houden. Evenmin is duidelijk of T cellen hierbij een rol spelen, en of exogene dan wel endogene antigenen van invloed zijn. Daarom werd besloten het ontstaan en de regulatie van de antilichaamdiversiteit te onderzoeken bij muizen van verschillende leeftijden, bij muizen met een T cel deficiëntie en bij 'kiemvrije' muizen. Deze laatste zijn nog nooit met microörganismen in aanraking geweest. Bovendien werden de door ons gebruikte kiemvrije muizen gevoed met een synthetisch dieet en in speciale plastic blazen grootgebracht om stimulatie met antigenen van buitenaf uit te sluiten. Voorts werden jonge voorlopercellen van de B lymfocyten, de zogenaamde pre-B cellen, uit het beenmerg van muizen geïsoleerd om ze buiten het lichaam *('in vitro')* te laten uitgroeien tot klonen antilichaamproducerende cellen. De resultaten van deze experimenten werden vergeleken met die verkregen met op normale wijze in het dier *('in vivo')* ontstane B lymfocyten. Van alle hierboven genoemde groepen muizen werden de milt- en beenmergcellen *in vitro* gekweekt om het B cel repertoire, zoals dat dagelijks wordt aangemaakt, te onderzoeken. Daarnaast werd in dezelfde organen het aantal en het repertoire bepaald van de 'spontaan' voorkomende antilichaamproducerende cellen, die waarschijnlijk al een selectie hebben moeten doormaken om tot dit stadium te komen. De resultaten van deze onderzoekingen zijn beschreven en bediscussieerd in de hoofdstukken IV-XII van dit proefschrift.

ABBREVIATIONS

B cell	Bone marrow-derived lymphocyte
BM	Bone marrow
BSA	Bovine serum albumin
BSS	Balanced salt solution
C_H	Constant region of the immunoglobulin heavy chain
C_L	Constant region of the immunoglobulin light chain
Cμ	Cytoplasmic μ chains
CD	Chemically defined
cIg	Cytoplasmic immunoglobulin(s)
CV	Conventional
D	Diversity region of the immunoglobulin heavy chain
DNA	Deoxyribonucleic acid
DNP	Dinitrophenyl
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
FITC	Fluorescein isothiocyanate
GF	Germfree
GRBC	Goat red blood cell(s)
Gy	Gray (1 Gy = 100 rad)
HRBC	Horse red blood cell(s)
IEF	Isoelectric focussing
IF	Immunofluorescence
Ig	Immunoglobulin(s)
J_H	Joining region of the immunoglobulin heavy chain
J_L	Joining region of the immunoglobulin light chain
LPS	Lipopolysaccharide
2ME	2-Mercaptoethanol
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
NI	Natural ingredient
NIP	4-Hydroxy-5-iodo-3-nitrophenyl
NNP	4-Hydroxy-3,5-dinitrophenyl
NP	4-Hydroxy-3-nitrophenyl
PBS	Phosphate buffered saline
PFC	Plaque-forming cell(s)
PWM	Pokeweed mitogen
RELISPOT	Reverse enzyme-linked immunospot assay
RIA	Radioimmunoassay
RNA	Ribonucleic acid
sIg	Surface immunoglobulin(s)
SRBC	Sheep red blood cell(s)
T cell	Thymus-derived lymphocyte
TDL	Thoracic duct lymphocytes
TNP	2,4,6-Trinitrophenyl
TRITC	Tetramethylrhodamine isothiocyanate
V_H	Variable region of the immunoglobulin heavy chain
V_L	Variable region of the immunoglobulin light chain

CHAPTER I

GENERAL INTRODUCTION

As opposed to more primitive organisms, vertebrates possess an immune system based on a high degree of *specific* recognition and memory of the agent which elicits the immune response. This system is superimposed on, and integrated into, the phylogenetically older *unspecific* defense mechanisms. The requirement of specificity limits the components of the immune system to lymphocytes and antibodies. Lymphocytes can be divided into two major classes, T cells and B cells, which occur in roughly equal numbers, 1×10^9 and 1×10^{12} of each in mouse and man, respectively. The following parts of this chapter will deal mainly with B cell differentiation and B cell heterogeneity, since the studies described in this thesis concern the B cell specificity repertoire.

I.1. Stages of B cell development

B lymphocytes are the precursors of antibody-secreting cells and each of them is precommitted to make immunoglobulin (Ig) molecules of a unique antigen-binding specificity. In mammals, B lymphocytes are produced in fetal and adult hematopoietic tissues (Owen et al., 1977; Melchers, 1979; Osmond, 1980a, b; Kincade, 1981; Osmond et al., 1981; Cooper et al., 1984). In adults, the bone marrow (BM) is the major site of the generation of immunocompetent B lymphocytes (Osmond 1980a, b; Osmond and Batten, 1984), which are thought to be derived from pluripotent hematopoietic stem cells (Kincade, 1981). Large lymphoid cells that contain cytoplasmic μ (cμ) chains probably represent the first detectable stage of B cell differentiation in, for example, mouse (Raff et al., 1976; Owen et al., 1977; Osmond, 1984; Osmond and Owen, 1984) and man (Gathings et al., 1977; Janossy et al., 1980; Cooper, 1981; Kamps and Cooper, 1982; Hokland et al., 1983). These cμ positive ($c\mu^+$) cells, called pre-B cells, do not express IgM on their cell surface, but may react with peanut agglutinin (Osmond, 1984; Osmond et al., 1984), or with some monoclonal antibodies directed against cell surface antigens (Coffman, 1982; Landreth et al., 1983; Melink and LeBien, 1983). Some of these antigens are not only present on most surface IgM positive ($sIgM^+$) cells but, interestingly, also on a substantial number of the lymphoid cells in the BM which do not synthesize Ig chains, indicating that these cells might represent progenitors of pre-B cells (Landreth et al., 1983; Kincade et al., 1984; McKearn et al., 1984). However, this was not confirmed for pre-B cells in fetal liver (Velardi and Cooper, 1984), suggesting that at the moment, the demonstration of rearrangement and expression of Ig genes, a prerequisite to produce Ig, may be required for the precise identification of cells belonging to the B lineage, both in ontogeny and early adult differentiation (Coffman and Weissman, 1983). Many of the large $c\mu^+$ cells are cycling and give rise to small $c\mu^+$ lymphocytes (Landreth et al., 1981; Opstelten and Osmond, 1983; Osmond and Owen, 1984). The latter cells subsequently start to express IgM molecules on their cell surface, a process accompanied or even preceded by the expression of other cell surface markers (Kincade et al., 1981; McKenzie and Zola, 1983; Rosenthal et al., 1983; Hofman et al., 1984; Velardi and Cooper, 1984). Some of these markers are identified as receptors for growth and differentiation factors (e.g., mitogens and T cell derived substances), others as receptors for the Fc portion of Ig molecules (Fc receptors) or as receptors for complement (Vitetta et al., 1984). Finally, B cells can migrate from the BM via the bloodstream to spleen and lymph nodes (Osmond, 1980a, b)

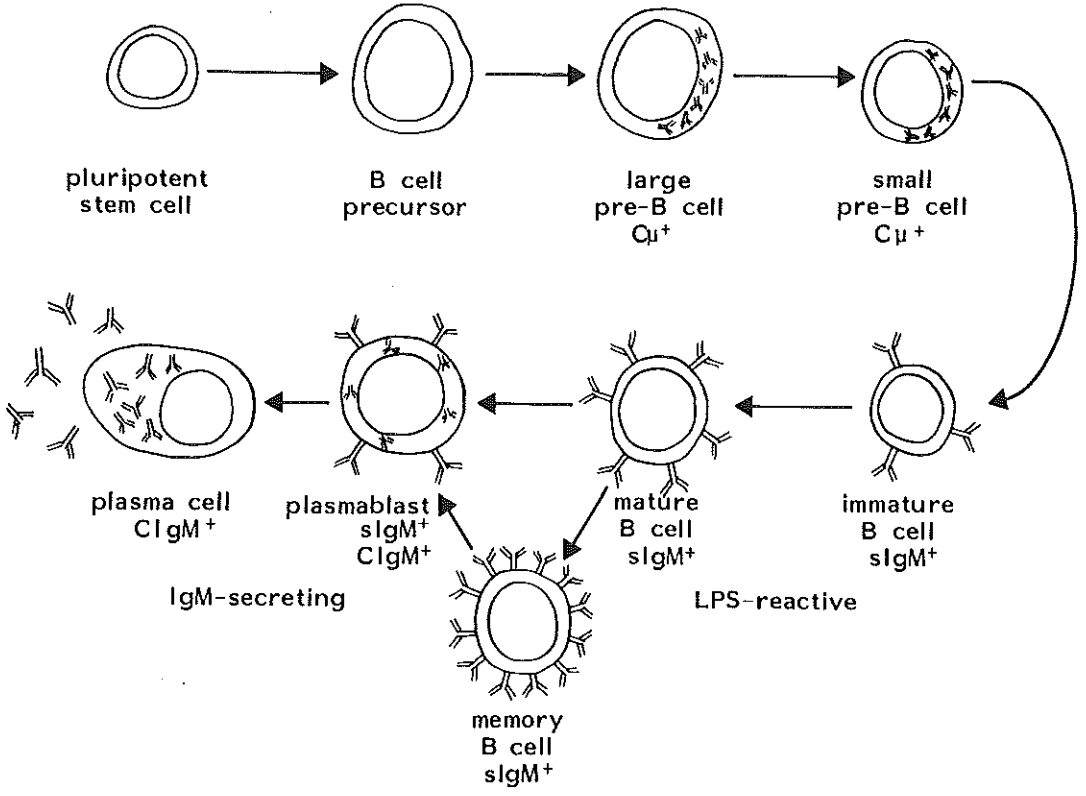

Figure 1. Hypothetical B cell differentiation scheme: from stem cell to IgM-secreting plasmacell. During B cell development other Ig-isotypes than IgM may be expressed on the cell surface and subsequently secreted (modified by Calvert et al., 1984).

and can be activated by antigen or mitogen to clonal proliferation and differentiation in complex processes, probably involving macrophages and T cells and the soluble products they can release (Ballieux and Heijnen, 1983; Corbel and Melchers, 1984; Coutinho et al., 1984b; Howard et al., 1984; Kehrl et al., 1984; Kishimoto et al., 1984; Melchers and Andersson, 1984; Melchers et al., 1984; Muraguchi et al., 1984). A schematic representation of the B cell development is given in Figure 1.

Many of the data pertinent to B cell development were not only obtained in healthy laboratory animals and humans, but also in animal models and human cases of immunodeficiency or immunoregulatory diseases (Pearl et al., 1978; Cooper, 1981; Kincade et al., 1982; Landreth et al., 1984). In addition, the phenotypic, functional and Ig gene rearrangement analyses of *in vitro* transformed lymphoid cell lines and of cells and cell lines derived from animals and patients with leukemia or malignant lymphoma have been of great value for insight into B cell differentiation (Janossy et al., 1980; Knapp, 1981; Abbas, 1982; Foon et al., 1982; Paige et al., 1982; Schroff et al., 1982; Sugiyama et al., 1982; Korsmeyer et al., 1983; Anderson et al., 1984; Bernard et al., 1984; Korsmeyer and Waldmann, 1984; Waldmann, 1984).

I.2 Immunoglobulin isotype switch

The effector function of the B cell system is eventually performed by the Ig produced by its Ig-secreting cells. 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 V_L part is coded by a variable (V) and a joining (J) element, while the V_H part is coded by a V, a diversity (D) and a J element (Figure 2). The constant part determines the Ig (sub)class or

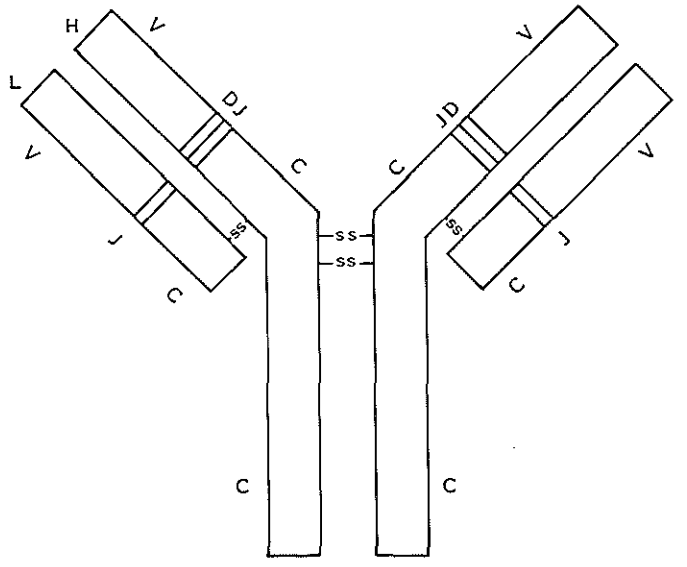

Figure 2. Schematic representation of an Ig molecule consisting of two heavy (H) and light (L) chains. Each L chain is built up from a constant (C), joining (J) and variable (V) part and every H chain consists of a C, J, diversity (D) and V part. Both heavy chains and light chains are linked together with disulfide (S-S) bonds.

isotype (e.g., in the mouse IgM, IgD, IgG3, IgG1, IgG2b, IgG2a, IgE and IgA) and the V, D, and J parts, the antibody specificity for antigen (Edelman, 1973; Jeske and Capra, 1984). It has been generally accepted that $sIgM^+$ B cells give rise to cells synthesizing other Ig isotypes (Cooper et al., 1980), still expressing the same specificity (Press and Klinman, 1973; Gearhart et al., 1980) and the same idiotype (Gearhart et al., 1975) as their ancestors. By this mechanism, called Ig class switching or isotype switch, the full range of antibody specificities can be expressed in molecules having different biological functions (Jeske and Capra, 1984). In the mouse the order of the C_H genes, which are juxtaposed 5' to the V_H genes, is 5'-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα-3' (Honjo 1983; Honjo et al., 1983) and the switching process is known to occur through a complex set of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) splicing and rearrangement events (Honjo 1983; Honjo et al., 1983; Shimizu and Honjo, 1984), involving either deletion of the DNA sequences between the V_H and C_H gene used (Honjo and Kataoka, 1978; Radbruch and Sablitzky, 1983), or sister chromatid exchange (Obata et al., 1981). A schematic representation of the mouse Ig heavy chain genes and the rearrangement events necessary for μ- or γ1-synthesis are given in Figure 3.

I.3. B cell antibody specificity repertoire

B lymphocytes are destined to produce Ig molecules with a unique antigen-binding specificity. 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 *not instructed* to produce antibodies by antigen, but rather, already existing B cells are *selected* by antigen via their surface Ig receptors to proliferate and produce specific antibodies. Since the organism is able to make antibodies against antigens which it has never encountered before, this implies that the B cell system must be capable of synthesizing a vast array of antibodies with various specificities. Extensive studies of the Ig genes that code for the Ig molecules have revealed how mouse and human in principle are able to generate such a large repertoire of antibody specificities (Tonegawa, 1983; Max, 1984). During the development of B cells to mature Ig-producing cells, the separated gene segments, coding for the variable and constant regions of the Ig heavy and light chains, are joined by a process that probably results in the loss of the intervening DNA sequences. The rearranged V gene, which thereby has become located in the proximity of the C gene, is transcribed as a single unit together with the C gene. The non-coding intervening sequences between the V gene complex and the C gene is then spliced during the processing of the primary RNA transcripts to form mature messenger RNA, resulting in heavy and light chain production, eventually leading to the production of Ig molecules (Tonegawa, 1983; Wall and Kuehl, 1983; Max, 1984).

The multiplicity of the germline V_H and V_L genes and the somatic recombinational processes involved in the formation of complete Ig genes as well as the putative random association of heavy and light chains can account for the diversity of the antibody repertoire (Tonegawa, 1983). Moreover, there is evidence for high mutation rates of V genes, which might accompany the clonal expansion of immunocompetent cells and might contribute to even more antibody diversity (Staudt and Gerhard, 1983; Tonegawa, 1983). It has been suggested (Bothwell et al., 1981; Gearhart et al., 1981) that somatic mutations may be linked to switch recombinations since V_H regions of μ chains exhibit far fewer mutations than those of γ chains, which are expressed later in development. There is, however, also evidence against this concept (Tonegawa, 1983).

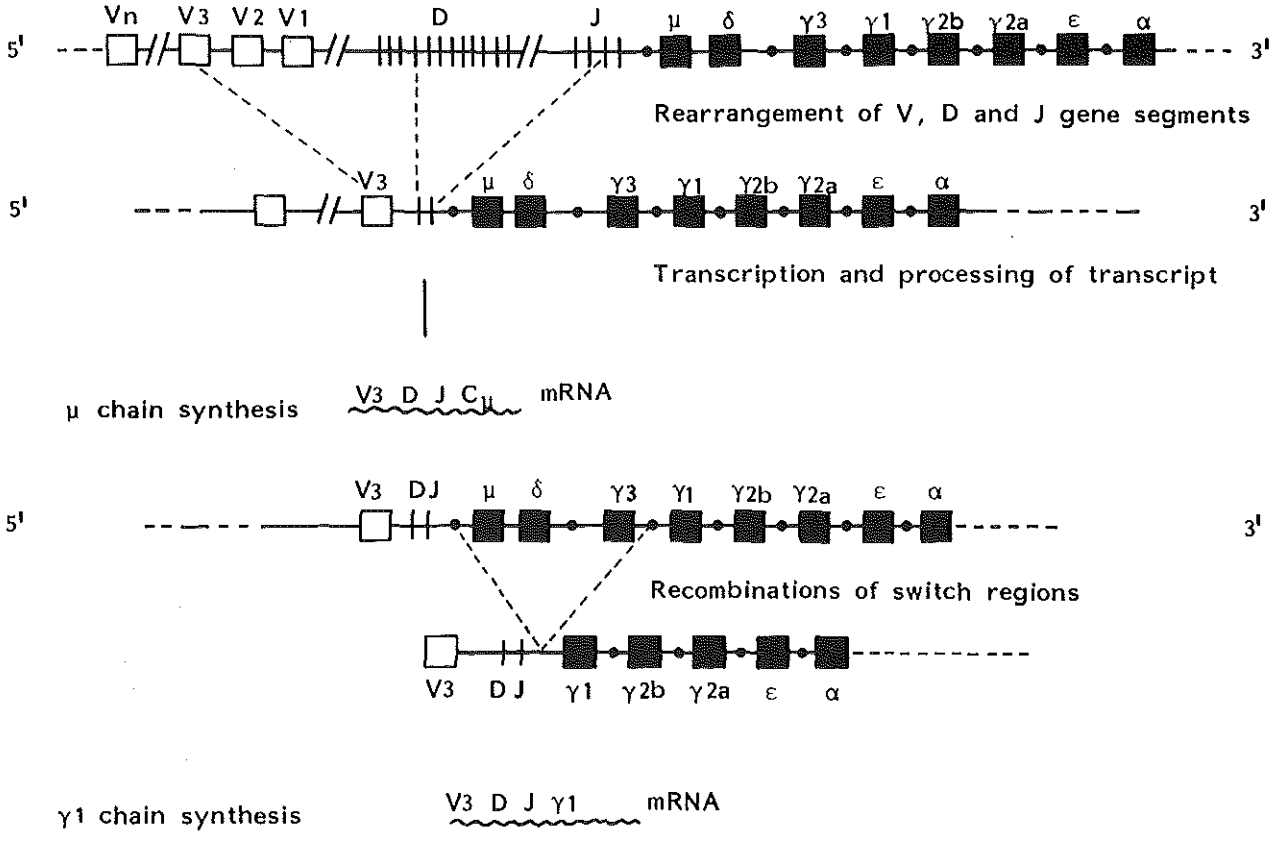

Figure 3. Schematic representation of the mouse Ig heavy chain gene segments and the processes leading to μ chain synthesis and the synthesis of γ 1 chains according to the deletion model (modified from Honjo, 1983 and Calvert et al., 1984).

The total number of different Ig molecules that the immune system of an individual produces is often called the antibody specificity repertoire or B cell repertoire. However, the B cell repertoire can be more precisely defined by subdividing it into three classes taking into account the various B cell differentiation stages (Coutinho et al., 1984a):

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.
2. The *available* repertoire defined as the set of diverse antibody molecules that are expressed by immunocompetent but resting B lymphocytes.
3. The *actual* repertoire represented by that set of antibodies produced by B cells that at any moment are activated to do so.

I.4. The network theory

The netwerk theory, originally formulated by Jerne (1973, 1974), has profoundly influenced current thoughts on the possible regulation mechanisms involved in the establishment of available and actual B cell repertoires. This describes the immune system as an enormous and complex network of antibodies recognizing and therefore influencing each other, leading to an autonomous steady state of activity ('eigen-behaviour'), even in the absence of antigens, in the classical sense.

In more detail, individual Ig molecules are considered to express two sets of sites with which they interact with other elements of the immune system. Each molecule bears a combining site (paratope) with which it interacts with antigenic determinants (epitopes) of conventional antigens and with other Ig molecules which possess one or more antigenic determinants, defined by the structure of their variable regions, 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. The antibodies expressing such idiotopes are called the internal image set or 'homobodies' (Lindenmann, 1973, 1979). Moreover, for each antibody expressing a given paratope and idiotope (or set of idiotopes) a complementary antibody exists possessing a paratope capable of binding to the idiotope of the first one and expressing its own set of idiotopes and so on. 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 as originally formulated (Jerne, 1974), has been subsequently extended to a unifying hypothesis postulating autonomy and completeness in the immune system embodied by the interaction between complementary molecules, including not only antibodies, but also T cell receptors (Coutinho, 1980), receptors controlling the growth and differentiation of B cell precursors and the activation of mature B lymphocytes (Coutinho, 1980; Coutinho et al., 1983) and finally all other structures in the organism which are available for interactions with the immune system (Coutinho, 1984; Coutinho et al., 1984a). Recently, Jerne reconsidered some basic questions related to the function of idiotypic networks in the immune system (Jerne, 1984). In spite of this inspiring concept, reflected by the abundance of experimental data and model systems concerning idiotypic-anti-idiotypic interactions (Eichmann, 1978; Bona, 1981; Bona and Cazenave, 1981; Janeway et al., 1981; Urbain et al., 1981; Paul and Bona, 1982; Urbain and Wuilmart, 1982a, b; Westen-Schnurr, 1982; Bona and Köhler, 1983; Rajewski and Takemori, 1983; Köhler et

al., 1984; Möller, 1984) the basic rules governing the internal activity of the immune system which includes the selection of repertoires remain largely unknown. Recently a formal proof of the existence of an idiotype-anti-idiotypic network was given, although no evidence could be provided for the function of the interactions detected (Coutinho et al., 1984a; Holmberg et al., 1984).

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CHAPTER II

ASSAY SYSTEMS FOR B CELL DIFFERENTIATION

As stated before, every B cell is committed to produce Ig of a particular specificity and with the unraveling of the structure and functioning of the Ig genes, a new tool for B cell differentiation research has become available (Joho et al., 1983). It is now possible, using recombinant DNA techniques, to investigate whether cells possess rearranged Ig genes and at which differentiation stage such rearrangements take place. In addition, it is feasible to correlate these features with the phenotype of the cells as expressed by membrane antigens, which can be detected by for example, monoclonal antibodies. The problem of having too few cells to perform these DNA analyses might partly be overcome by the analysis of B cell tumors or tumor cell lines expressing one or various differentiation stages (Korsmeyer et al., 1983). The improvement of *in vitro* culture techniques allows the stimulation of such cells which permits the monitoring of changes in their gene rearrangements and phenotype (Abbas, 1982; Nadler et al., 1982; Sugiyama et al., 1982, 1983). The mechanism of Ig isotype switch was studied in this way (Alt et al., 1982; Honjo et al., 1983). However although studies of myeloma, plasmacytoma and B cell lymphoma cell lines may be interesting, it is obvious that not all aspects of B cell differentiation can be covered by solely studying these tumor lines, which might display some artefacts since they are transformed. Therefore, the analysis of B cell development in different organs and performed at the cellular level under normal *in vivo* or controlled *in vitro* conditions is most important. In particular, the search for relatively rare (sub)populations and the ability to culture and analyze these under clonal conditions allowed some estimates of the frequency of their occurrence and their functional capacities. These rare cell populations might be enriched by e.g. fluorescence activated cell sorting, 1 g velocity sedimentation (a cell separation method which separates cells on the basis of their size), buoyant density centrifugation and rosetting or cell adherence techniques. Since B cells will produce antibodies, it is obvious that analysis of antibody-forming potencies has been widely used as a screening system for B cell activity. Ig production or secretion by B cells can be measured using radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA), isoelectric focussing (IEF) or at the single cell level by immunofluorescence (IF) techniques or plaque assays, which can visualize the Ig production by single cells. In particular the analyses of the B cell specificity repertoire have been greatly improved by the development of assay systems analyzing B cells and their products at the clonal level. Some of the clonal assay systems (II.1-II.4) and some assay systems for the detection of single Ig-secreting cells (II.5) will be discussed in more detail.

II.1 In vitro splenic focus assay

The *in vitro* splenic focus assay has been developed by Klinman and coworkers (Klinman and Aschinazi, 1971; Klinman, 1972; Klinman and Press, 1975; Sigal and Klinman, 1978) to analyze the functional capacities of B cells and B cell precursors at the clonal level. Usually, limiting numbers ($0.5\text{-}4 \times 10^6$) of donor spleen cells are intravenously injected into lethally irradiated syngeneic mice which have been preimmunized with a carrier protein to provide maximal T cell help (thought to be relatively radio-resistant) for the injected B cells to be assayed. Approximately 5% of the injected B cells are present in the recipient spleen when it is removed 16 hours later. The

spleen is then cut into about 50 fragments, which are placed in individual microcultures with a hapten-carrier conjugate. After 7-11 days antibody is measured in the culture supernatants by RIA or IEF and the Ig-producing cells can then be analyzed by cytoplasmic IF or by a relevant plaque assay. The B cells that are detected in this splenic focus assay are of all sizes, as was indicated by cells injected after velocity sedimentation separation. It was estimated that about 80% of the DNP-specific B cells that lodge in the irradiated spleens can be stimulated to antibody secretion with DNP-hemocyanin (Klinman et al., 1976). The method detects B cells in fetal liver and in neonatal spleen (Press and Klinman, 1973; Teale and Mandel, 1980), while surface Ig negative B cell precursors have been revealed with this assay in adult BM (Klinman et al., 1983; Riley et al., 1983). Moreover, it has been successfully applied to the analysis of Ig isotype switching at the clonal level (Gearhart et al., 1975; Gearhart, 1977; Gearhart et al., 1980; Gearhart and Cebra, 1981) and to the analysis of B cell repertoires of fetal, neonatal, young adult and old normal mice, nude mice and germfree mice (Press and Klinman, 1974; Sigal et al., 1975; Cancro and Klinman, 1980; Klinman et al., 1983; Zharhary and Klinman, 1983, 1984).

II.2 In vitro limiting dilution assay

Lefkovits was the first who devised an assay in which B cells are diluted in cultures containing antigen and constant numbers of accessory and filler cells (Lefkovits, 1972, 1979; Lefkovits and Waldmann, 1979, 1984; Waldmann and Lefkovits, 1984). Many small aliquots of lymphoid cells are cultured under conditions that allow the growth and maturation of every reactive B cell into a clone of Ig-secreting cells. Thus the analysis is conducted with such a range of cell concentrations that only the titrated B cells are limiting for the reaction. The number of cells in each aliquot is chosen so that a considerable fraction of cultures will not contain any precursor cell. From the fraction of non-responding cultures, using the Poisson formula, it is possible to calculate the frequency of precursor cells (Lefkovits and Waldmann, 1979, 1984). The analysis of the fraction of non-responding cultures can be performed on the culture fluid (e.g. by the hemolytic spot test) or on the cultured cells themselves (by plaque-forming cell assays).

Instead of antigen, mitogens are often used to estimate the frequency of reactive cells (Andersson et al., 1976, 1977a, b, c). Normal, newly-formed B cells can be stimulated to growth by mitogens such as LPS (Andersson et al., 1972, 1973; Rusthoven and Phillips, 1980; Freitas et al., 1982), that circumvents the binding-step to surface-bound Ig, and therefore stimulates the B cells irrespective of their V-region specificity. Mitogens are thought to bind directly to their putative cell surface-located receptors, which are polyclonally distributed over the antigen-specific B cells (Vitetta et al., 1984). Binding of mitogen is the signal for a lymphocyte to undergo a set of not precisely known reactions (Ashman, 1984), eventually leading to growth and maturation. A prerequisite for this *in vitro* growth of normal B lymphocytes is the use of suitable culture conditions, which allow every single lymphocyte, having the capacity to be stimulated by the mitogen employed, to grow and develop into a clone of cells. Growth requires, in addition to RPMI 1640 medium and the presence of a mitogen, growth-supporting fetal bovine serum and 2-mercaptoethanol (2ME), while for maturation, mouse or rat thymus filler cells are necessary (Andersson et al., 1976, 1977a, b, c). Serum-free media have also been used (Iscove and Melchers, 1978). Recently it was reported that macrophages are a prerequisite for the *in vitro* activation of B cells by LPS (Corbel and Melchers, 1983; Fernandez and Severinson, 1983;

Melchers and Corbel, 1983). In general, every third murine splenic B cell is responsive to LPS and will grow up to 7 days, dividing every 18 hours under these *in vitro* culture conditions. Cultures are normally analyzed on day 5 for IgM secretion and on day 7 for the secretion of IgG or IgA in the appropriate plaque assays. For the analysis of supernatants in RIA or ELISA the cells are generally cultured for up to 11 days. In some instances B cells could be grown for two to three weeks, but these lines were then invariably lost (Melchers et al., 1975; Andersson et al., 1976), probably due to terminal differentiation into a non-dividing type of lymphoid cell such as a plasma cell. However, some reports of continuously growing normal B cell lines of mouse (Howard et al., 1981; Whitlock and Witte, 1982; Whitlock et al., 1983, 1984) or human (Sredni et al., 1981) origin have been published. In addition, mouse pre-B cell clones have been established recently (Denis et al., 1984; Palacios et al., 1984).

The *in vitro* limiting dilution assay has been successfully applied to the assessment of the diversity of antibody specificities among mitogen-responsive B cell populations in spleen (Andersson et al., 1977c; Eichmann et al., 1977) and BM (Benner et al., 1981a) and to the measurement of the number of LPS-reactive B cells among the progeny of *in vitro* differentiated B cell precursors (Lau et al., 1979; Deslauriers-Boisvert et al., 1980; Juy et al., 1983; Nishikawa et al., 1983). Moreover, the method was used to study the Ig isotype switch at the clonal level (Andersson et al., 1978; Coutinho and Forni, 1982) and to determine the numbers of LPS-reactive B cells in different organs during ontogeny (Melchers, 1977, 1979; Melchers and Abramczuk, 1980). The limiting dilution culture system has also been used to analyze human peripheral blood B cells for mitogen reactivity, class switch and specificity repertoire (Stevens et al., 1981; Martinez-Maza and Britton, 1983; Yarchoan et al., 1983; Yarchoan and Nelson, 1984).

II.3 Semisolid agar cloning

Murine B cells are able to form colonies in semisolid agar cultures when cultured under the appropriate conditions adding 2ME, SRBC, LPS or adherent layers of peritoneal exudate macrophages to the culture medium (Kincade, 1981). Up to 13% of the B cells form colonies, as a result of 5 or more cell divisions (Kurland et al., 1977). The clonable cells are as heterogenous as all other B cells with respect to physical properties. The only defined B cell population that has been found to totally lack colony-forming cells is found in partially immunodeficient CBA/N mice. In general, the stage of Ig secretion is not often reached in B cell colonies (Kincade, 1981), although some successful attempts have been made to optimize the culture conditions to study for example the diversity of antibody specificities (Paige and Skarvall, 1982) or the differentiation of B cell precursors (Jyonouchi and Kincade, 1983; Paige, 1983; Paige et al., 1984).

II.4 Ig-secreting hybridomas

The 'spontaneously' occurring Ig-secreting cells, the terminus of B cell differentiation, have until recently been studied by IF and plaque assays detecting the secretion of antigen-specific Ig or Ig of several subclasses (Benner et al., 1981b, c, 1982). However, it is not possible to further analyze these antibody-secreting cells, e.g. for detailed characterization of their antibodies or the structural analysis of their V-region and of the genes by which they are encoded. For that purpose, large numbers of cells producing that type of antibody are necessary. A way to overcome this problem is to construct hybridomas of e.g. spleen cell suspensions derived from nor-

mal un-immunized mice to immortalize cells expressing a given pair of V regions. As only activated lymphocytes will form stable hybrids with the fusion partner, it is assumed that such collections of hybridomas represent cells that had been stimulated in the internal environment and constitute the actual repertoire of the normal mouse at that time (Coutinho et al., 1984). The hybridoma technology has been successfully employed to study the specificity repertoire of 'spontaneously' occurring ('background') Ig-secreting cells (Dighiero et al., 1983; Holmberg et al., 1984) as well as in the study of the repertoire in auto-immune mice (Pages et al., 1978; Andrzejewski et al., 1980). Moreover, the variations within the repertoire of antibody secreting cells that have responded to specific antigenic challenges (Staudt and Gerhard, 1983) or to polyclonal activation by LPS (Andersson and Melchers, 1978) have been studied to establish the diversity of the available antibody repertoires at different stages in ontogeny (Kearney et al., 1981). Finally, such studies have been performed on hybridomas made with fetal liver-derived pre-B cells (Kearney et al., 1981).

II.5 Assays for the detection of single Ig-secreting cells

II.5.1 Antigen-specific plaque assay

The hemolytic plaque assay has been developed by Jerne and Nordin (1963) and the methodology and theory have been extensively described (Jerne et al., 1974). In brief, lymphoid cells are mixed with a suspension of sheep red blood cells (SRBC) and immobilized in a gel (agar) or in a liquid medium enclosed in a sealed chamber according to the slide modification of Cunningham and Szenberg (1968). Specific antibody synthesized by some of the lymphoid cells is released and diffuses from these cells. The antibody is trapped by the red blood cells in the areas immediately surrounding the antibody-secreting cells. In the presence of complement, which will bind to the antibodies coating the red blood cells, these red blood cells will lyse. As a result, a clear area - a plaque - forms around each antibody-producing cell, which is now called a plaque-forming cell (PFC). Examination under the microscope reveals that, in general, one plaque corresponds to one PFC and therefore the total number of antibody-secreting cells in the suspension can be determined. Instead of SRBC, red blood cells of other animals can be used as antigens or the SRBC can be modified by coupling other antigenic determinants to them, for example haptens as 4-hydroxy-5-iodo-3-nitrophenyl (NIP), 4-hydroxy-3,5-dinitrophenyl (NNP) or 2,4,6-trinitrophenyl (TNP), allowing the enumeration of various antigen-specific PFC.

In this so-called direct plaque assay, the only antibodies detected are of the IgM class, since IgG, IgA and IgE antibodies are not efficient enough in complement binding to mediate cell lysis under these experimental conditions. To detect these antibodies of non-IgM isotype, the indirect plaque assay has to be applied. In the latter assay, prior to the addition of complement, an IgG-, IgA- or IgE-specific rabbit antiserum is added, which will bind to antibody of the relevant Ig class produced by the cell suspension and bound to the indicator erythrocytes. The class specific antibodies cross-link the coating antibodies and generate conditions for efficient complement binding and hence for cell lysis. Since also direct plaques will develop, the number of such 'facilitated' or 'indirect' plaques can in principle be determined by subtracting from the number of plaques developed with the antiserum, the number of plaques obtained in its absence (Jerne et al., 1974).

II.5.2 Reverse plaque assay

In the reverse plaque assay, developed by Molinaro and Dray (1974), SRBC are directly coated with anti-Ig antibodies by chromiumchloride. In this assay Ig-secreting cells form hemolytic plaques independently from the antibody specificity of the secreted Ig. This assay can therefore be applied to enumerate subpopulations of Ig-secreting cells on the basis of the isotype or allotype they secrete.

II.5.3 Protein A plaque assay

The protein A plaque assay, developed by Gronowicz et al. (1976), is based on the property of protein A from the cell wall of *Staphylococcus aureus* to bind to the Fc portions of IgG molecules, especially to those of rabbit origin. Protein A coated erythrocytes are used as indicator and mixed with a lymphoid cell sample, containing Ig-secreting cells, complement and the IgG fraction of a rabbit antiserum specific for a particular Ig (sub)-class. Complexes of the secreted Ig and IgG antibodies specific for the secreted Ig bound to protein A on the red cell surface, activate sufficient amounts of complement to lyse the protein A-coated red cells. Thus, using only one type of indicator red cells and various (sub)class specific antisera of rabbit origin, Ig-secreting cells of various isotypes can be enumerated regardless of the specificity of the secreted antibodies. Since its introduction, the protein A plaque assay has been successfully employed for a variety of species including man (Burns and Pike, 1981; Burns et al., 1982; Librach and Burns, 1983; Jones, 1983).

II.5.4 Solid-phase enzyme-linked immunospot (ELISPOT) assay

Recently a solid-phase enzyme-linked immunosorbent assay (ELISPOT) has been described for the detection of antibody-secreting cells (Sedgwick and Holt, 1983; Czerkinsky et al., 1983). This technique is based upon the principles of ELISA. In short, single cell suspensions of antibody-secreting cells are incubated on a solid phase (e.g. polystyrene plates) to which specific antigen has been chemically conjugated. Antibody attaches to the latter within the immediate microenvironment of the antibody-secreting cell, producing localized zones of bound antibody, which are subsequently developed as visual 'spots' in the ELISA. The ELISPOT assay has a sensitivity and specificity at least equivalent to hemolytic plaque assays and provides a useful alternative to conventional antigen-specific plaque-forming cell assays by circumventing the difficulties often encountered in coupling antigens to erythrocytes.

II.5.5 Reverse enzyme-linked immunospot (RELISPOT) assay

A reverse modification of the ELISPOT assay has been recently described for the enumeration of Ig-secreting cells irrespective of their antigen specificity (Czerkinsky et al., 1984). Using the appropriate antiserum against mouse or human Ig the RELISPOT assay reveals similar numbers of mouse or human Ig-secreting cells as the protein A plaque assay (Czerkinsky et al., 1984).

II.6 References

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CHAPTER III

INTRODUCTION TO THE EXPERIMENTAL WORK

The process of B cell differentiation has been divided into antigen-independent and antigen-dependent phases. The earliest differentiation steps, leading to the development of clonally diverse sIg^+ B lymphocytes appear to be driven by local environmental influences. In contrast, B cell activation, proliferation and terminal plasma cell differentiation are thought to be initiated through contact with antigens and helper T cells or by polyclonal mitogens ingested as food substances or brought into the body by invasive microorganisms.

The antigen-independent differentiation of B lymphocytes has mainly been studied by examining their cellular markers and their acquisition of immunocompetence. In general, these investigations were performed at different sites of B cell development, in fetal as well as in young adult birds and mammals. In addition, the B cell antibody repertoire as expressed by the constant and variable Ig heavy and light chain genes has been studied extensively, by the structural analysis of both the antibodies and the genes by which they are encoded. In this way much information has been gained about the genetic mechanisms involved in the generation of the B cell specificity repertoire and in the switch from IgM to other isotypes. It has also been found that in nonimmunized mice 'spontaneously' occurring ('background') Ig-secreting cells exist in the various lymphoid organs and that their numbers vary with the genetic background, age and antigenic load of the mice (Benner et al., 1982). These cells represent the terminus of B cell differentiation and their presence might therefore be largely antigen-dependent.

Despite the accumulating data on B cell development, little is known about the regulatory mechanisms that establish the available and functionally expressed repertoire in the immunocompetent resting B cell compartment. One of the basic questions is whether exogenous and endogenous influences impose any selection on the functional expression of antibody specificities or whether diversity and available repertoires can result simply from hierarchic stochastic processes of Ig gene expression by differentiating B cell precursors (Coleclough, 1983; Mäkelä et al., 1983). Furthermore, what are the rules governing the establishment of the actual repertoire expressed by 'spontaneously' occurring Ig-secreting cells from the available repertoire? The purpose of the studies presented in this thesis, therefore, was to obtain more insight into the regulatory aspects that are involved in the functional expression of the Ig C and V genes in the various lymphoid organs of the mouse. To this end, frequency analyses of Ig heavy chain isotype secretion (C gene expression) and of the secretion of specific IgM antibodies (V gene expression) were performed among the progeny of *in vitro* differentiated pre-B cells. The same was done of *in vivo* generated, newly-formed mitogen-reactive B cells (available repertoire) and among the 'spontaneously' occurring ('background') Ig-producing cells (actual repertoire). The parameters studied include age, T cells and exogenous antigens. The latter became feasible since with the successful breeding of germfree (GF) mice fed an ultrafiltered solution of chemically defined (CD) low molecular weight nutrients, exogenous stimuli such as antigens and mitogens are reduced to a minimum never attained before (Pleasants et al., 1981; Wostmann et al., 1982; Pleasants, 1984). We will refer to these mice as GF-CD mice or 'antigen-free' mice.

Newly-formed B cells were studied at the clonal level in the LPS-driven *in vitro* limiting dilution culture assay, which stimulates about every third B cell to growth and maturation into a clone of Ig-secreting cells independent of Ig receptor recognition (Andersson et al., 1977a, b, c). Both the LPS-stimulated B cell clones and the background Ig-producing cells were analyzed for Ig isotype expression with the protein A plaque assay (Gronowicz et al., 1976) and for specific IgM-antibody production with antigen-specific plaque assays (Jerne et al., 1974). Therefore we started our studies with the optimization of the protein A plaque assay and the LPS culture system. These aspects have been described in Chapter IV and Chapter V of this thesis, respectively.

In Chapter VI it has been investigated whether there is any *in vivo* regulation of the repertoire expression during the differentiation of large pre-B cells into small mature B cells. Therefore, large pre-B cells were isolated by 1 g velocity sedimentation and allowed to differentiate *in vitro* into LPS-reactive B cells. This population was compared with *in vivo* differentiated small mature B cells with regard to the number of IgM-secreting cells they produce after *in vitro* stimulation with LPS, their capacity to switch to the secretion of IgG1 and IgG3 and the absolute frequencies of cells specific for differently haptenated SRBC.

The frequency determination of LPS-reactive B cells developing into clones that secrete various Ig classes is described in Chapter VII. Switching frequencies from IgM-secreting clones to IgG1-, IgG2a-, IgG2b-, IgG3- or IgA-secretion were determined in spleen, BM, mesenteric lymph nodes (MLN) and among thoracic duct lymphocytes (TDL) of BALB/c mice. Furthermore, such switch frequencies were determined for LPS-reactive B cells in the spleen of BALB/c athymic 'nude' mice to study the regulatory influence of autologous T lymphocytes on the switch frequency. In addition, the frequencies of background IgM-, IgG1-, IgG2-, IgG3 and IgA-secreting cells were determined in the spleen, BM and MLN of BALB/c thymus-bearing mice and in the spleen of BALB/c athymic 'nude' mice to establish putative *in vivo* T cell regulatory influences on the Ig isotype expression.

In Chapter VIII the frequencies of LPS-reactive B cells and a part of their antibody specificity repertoire were determined in the spleen and BM of mice of various ages to investigate the influence of aging on the available B cell repertoire and capacity of isotype switch. In addition, the specificity repertoire of the background Ig-secreting cells was analyzed in the spleen and BM of aging mice to determine whether the actual B cell repertoire is affected by aging.

The possible role that T cells might play in the generation and expression of the available and actual B cell repertoire was investigated in Chapter IX. To this end, a part of the antibody-specificity repertoire of LPS-reactive B cells was determined in the spleen and BM of young and old C57BL/Ka athymic 'nude' mice. The numbers of the background IgM-, IgG- and IgA-secreting cells as well as the background IgM antibody specificity repertoire were assessed in the spleen and the BM.

In Chapter X the frequency of LPS-reactive B cells, a part of their antibody-specificity repertoire and their capacity to switch from IgM to IgG1 secretion were determined in the spleen and BM of conventional (CV) and 'antigen-free' C3H/HeCr mice of various ages. These 'antigen-free' mice had been

GF reared and maintained and fed an ultrafiltered solution of CD low molecular weight nutrients.

The influence of exogenous stimuli on the actual B cell repertoire has been determined using also GF-CD mice (Chapter XI). The numbers of background IgM-, IgG- and IgA-secreting cells and a part of the IgM antibody specificity repertoire were assessed in spleen, BM and MLN of CV mice and 'antigen-free' mice that were reared and maintained as described above.

III.1 References

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CHAPTER IV

IMPROVEMENT OF THE PROTEIN A PLAQUE ASSAY FOR IMMUNOGLOBULIN-SECRETING CELLS BY USING IMMUNOGLOBULIN-DEPLETED GUINEA PIG SERUM AS A SOURCE OF COMPLEMENT

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In: J. Immunol. Methods 43:219-224 (1981)

SUMMARY

This paper describes a modification of the protein A hemolytic plaque assay for the enumeration of immunoglobulin (Ig)-secreting cells independent of antibody specificity of the Ig. This assay was originally developed by Gronowicz et al. (1976), and is based upon binding of the Fc portion of IgG to protein A. Ig-secreting cells are mixed with protein A-coated sheep erythrocytes, developing rabbit anti-Ig antiserum and guinea pig serum as a source of complement. This mixture is either pipetted between two microscope slides, or added to agarose and plated on a petri dish or microscope slide. The hemolytic plaques are enumerated after incubation at 37°C. Here we show that purification of the guinea pig complement over a Sepharose protein A column in order to eliminate the IgG fraction facilitates plaque formation. This modification reduces the incubation period required for plaque formation, and yields a higher number of, and more discrete plaques, than the original method.

INTRODUCTION

Cells secreting immunoglobulin (Ig) can be detected by the hemolytic plaque assay. The original Jerne-type plaque assay detects single cells which secrete IgM antibodies that bind to determinants on the target red blood cell (Jerne and Nordin, 1963), usually sheep erythrocytes (SRBC). These determinants may be naturally occurring red blood cell antigens, or determinants artificially coupled to the red cell surface (Jerne et al., 1974). Cells secreting antibodies of a class other than IgM may also be detected in this assay. Antibodies specific for that class may be added as developing antiserum (Dresser and Wortis, 1965).

Cells secreting Ig without known antibody specificity can also be assayed in a hemolytic plaque assay. Two systems have been developed: the reverse plaque assay (Molinaro and Dray, 1974) and the protein A plaque assay (Gronowicz et al., 1976). Both are completely dependent on the presence of anti-Ig antibodies on the surface of the target SRBC. In the reverse plaque assay the SRBC are directly coated with the antibody molecules by the $CrCl_3$ method (Gold and Fudenberg, 1967). In the protein A plaque assay this is done indirectly, the target SRBC being coated with protein A by $CrCl_3$ and antibodies of the IgG class added to a mixture of these indicator red cells, the Ig-

secreting cells to be tested, and guinea pig serum as source of complement. During incubation at 37°C, complexes of the IgG antibodies and the secreted Igs bind to the protein A on the red cell surface, and activate complement to lyse the protein A-coated SRBC.

The protein A plaque assay is recognized as a valuable and easy tool for assessing Ig-synthesizing cells, both in human and in animal systems. The method, however, has one important disadvantage: the cells have to be incubated for at least 6 h in order to develop the maximum number of plaques. We have modified the original method and show in this paper that elimination of the IgG fraction from the guinea pig serum greatly reduces the incubation period necessary, and produces a higher number of, and more discrete plaques, than the original method.

MATERIALS AND METHODS

Animals

Female BALB/c mice, 8 weeks old, were purchased from the Radiobiological Institute TNO, Rijswijk (ZH), The Netherlands. Female Lewis rats, 6 weeks old, were purchased from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands.

Cells

We have described in detail the preparation of cell suspensions from thymus and bone marrow (Benner et al., 1981). The cells were washed twice and resuspended in RPMI 1640 medium containing 5% fetal bovine serum, batch T190101S (Gibco Bio-Cult, Irvine, Scotland). Viable cells were counted by trypan blue exclusion.

Culture conditions

BALB/c bone marrow cells (4000 viable nucleated cells per culture) were cultured for 5 days together with 6×10^5 growth-supporting rat thymus cells and 50 µg/ml *Salmonella abortus equi* lipopolysaccharide (LPS) in 0.2 ml of fresh RPMI 1640 medium supplemented with L-glutamine (4 mM), pyruvate (0.1 M), penicillin (100 IU/ml), streptomycin (50 µg/ml), 2-mercaptoethanol (5×10^{-5} M) and fetal bovine serum, batch T190101S, specifically selected for growth-supporting properties and low endogenous mitogenic activity. The LPS was kindly prepared and provided by Dr. C. Galanos, Max-Planck Institut für Immunbiologie, Freiburg i. Br., F.R.G. The cultures were set up in Microtest II tissue culture plates (Costar 3596; Costar, Cambridge, MA, USA).

Complement

Guinea pig serum was used as a source of complement (lot no. 303232B, Behringwerke, Marburg/Lahn, F.R.G., and lot no. 44011020, Flow Laboratories, Irvine, Scotland). Both products were dissolved in distilled water and adsorbed with SRBC to prevent lysis of the target SRBC in the plaque assay due to the presence of naturally occurring anti-SRBC antibodies in the guinea pig serum. A sample of each batch of complement was passed through a Sepharose-protein A column (Pharmacia, Uppsala, Sweden), according to Goding (1978) to remove the protein A-binding proteins, mostly IgG.

Assay for complement hemolytic activity

The native guinea pig sera and the protein A-purified guinea pig sera were titrated for complement hemolytic activity in the standard system. The

sera were tested in a mixture containing 50 µl of a 2.5% suspension of SRBC sensitized with hemolysin, 50 µl of serially diluted serum, and 0.3 ml of Veronal buffer (pH 7.2). The samples were incubated for 30 min at 37°C. Controls included undiluted serum and Veronal buffer (100 and 0% lysis respectively) instead of diluted guinea pig serum. The hemolysis units per ml were calculated from the reciprocal of the serum dilution that gave virtually complete lysis.

Protein A plaque assay

Ig-secreting cells were assayed by the hemolytic plaque assay as described by Gronowicz et al. (1976), with some minor modifications. *Staphylococcus aureus* protein A (Pharmacia, Uppsala, Sweden) was coupled to SRBC with $CrCl_3 \cdot 6H_2O$. For coupling, 1 ml washed packed SRBC, 1 mg of protein A (dissolved in 1 ml 0.9% NaCl), 9 ml 0.9% NaCl, and 50 µl $CrCl_3 \cdot 6H_2O$ (0.05 M) were mixed and incubated for 1.5 h at 37°C, with shaking every 15 min. Thereafter the cells were washed 3 times with 0.9% NaCl, and resuspended in a balanced salt solution (BSS). The protein A-coated SRBC were used within 3 days of preparation. The protein A plaque assay was performed in BSS using Cunningham-type chambers as described by Lefkovits and Cosenza (1979). Each chamber contained 100 µl of an appropriately diluted culture of LPS-activated bone marrow cells (washed in BSS before use), mixed with 20 µl of an optimal amount of diluted guinea pig complement (either native or purified over a Sepharose-protein A column as described above), 15 µl of an optimal amount of diluted specific rabbit-anti-mouse-IgM serum (kindly prepared and provided by Ms. L. Forni from the Basel Institute for Immunology, Basel, Switzerland), and 25 µl of a 10% suspension of protein A-coated SRBC. The rabbit antiserum used was purified over a Sepharose-protein A column as described by Goding (1978), in order to isolate the protein A-binding fraction of Ig. The chambers were incubated at 37°C for 10 h. The Ig-secreting plaque-forming cells (PFC) were counted under a dissecting microscope by dark field illumination.

RESULTS AND DISCUSSION

Ig-secreting cells were produced by activation of mouse bone marrow cells in bulk cultures, using LPS as a polyclonal activator. Cells harvested from 6 individual cultures were tested in the protein A plaque assay, using 4 different batches of complement: (1) normal Behringwerke guinea pig serum; (2) Behringwerke guinea pig serum purified over a Sepharose-protein A column to deplete the IgG fraction; (3) normal Flow Laboratories guinea pig serum; and (4) Flow Laboratories guinea pig serum purified over a Sepharose-protein A column. Thus, cells from each culture were tested with each of the 4 batches of complement separately. From each batch the same, optimal, number of hemolysis units of complement was used per assay. In each chamber the number of hemolytic plaques was counted at 30, 45, 60, 75 and 90 min, and at 2, 3, 4 and 5 h of incubation at 37°C. At each time point individual slides were taken from the incubator, counted at room temperature and immediately incubated again.

Fig. 1 shows the results obtained with the four batches of complement used. Visible plaques appeared faster with Flow complement (Fig. 1, right) than with Behringwerke complement (Fig. 1, left). This was especially true for the unpurified batches of complement. The mean number of plaques counted in the slides with the purified complement was greater than in the slides with the normal guinea pig serum. For the Behringwerke complement this diffe-

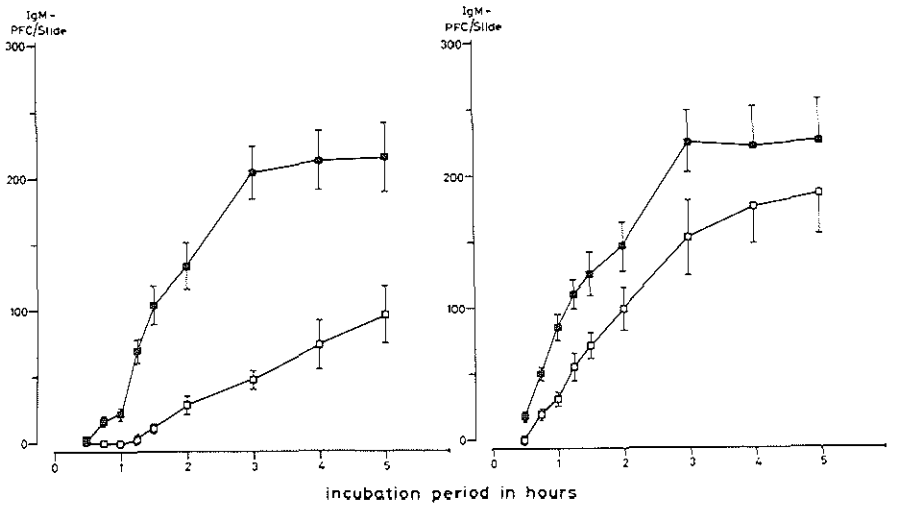

Figure 1. Number of IgM-secreting PFC per slide developed by Behringwerke (left) and Flow Laboratories (right) complement after various incubation (37° C) periods. The closed squares represent the mean number of PFC obtained with complement purified over a Sepharose-protein A column, the open squares the mean number obtained with normal guinea pig serum. Cells were obtained from bulk cultures of bone marrow cells activated by LPS. After 5 days of culture, the cells from each well were harvested, washed and resuspended in 2 ml of BSS. From this volume, 100 µl was tested per slide. Bars represent 1 S.E.M.

rence was significant at all time points tested; for the Flow complement this was the case only during the first 3 h of incubation. The mean number of plaques counted in the slides with purified Behringwerke and purified Flow complement was not significantly different at 90 min incubation or later. When the incubation period was extended to 10 h the number of plaques increased further in the slides with normal Behringwerke complement, but hardly or not at all in the other groups (data not shown). Similar results to those shown in Fig. 1 for development of plaques by IgM-secreting cells were obtained for the rates of appearance of plaques by IgG1-, IgG2-, IgG3- and IgA-secreting cells (data not shown).

The appearance of the plaques developed by normal guinea pig serum and purified complement was also clearly different. With purified complement larger and more discrete plaques developed. This was found with Behringwerke as well as with Flow complement and in Fig. 2 is illustrated for the latter.

Addition in the plaque assay of an amount of purified mouse IgG2b/κ (from MOPC 195) equivalent to the amount of IgG present in unpurified guinea pig serum reduced both the number of plaques developing and their size (data not shown). This emphasizes that other protein A-binding IgG can competitively inhibit the binding of the specific rabbit anti-Ig antibodies via the protein A moiety to the target SRBC. This problem is inherent in the protein A plaque assay and can be overcome only by choosing hypoglobulinemic guinea pigs as donor for complement, or by a simple run of the guinea pig serum over a Sepharose-protein A column. In the reverse plaque assay described by

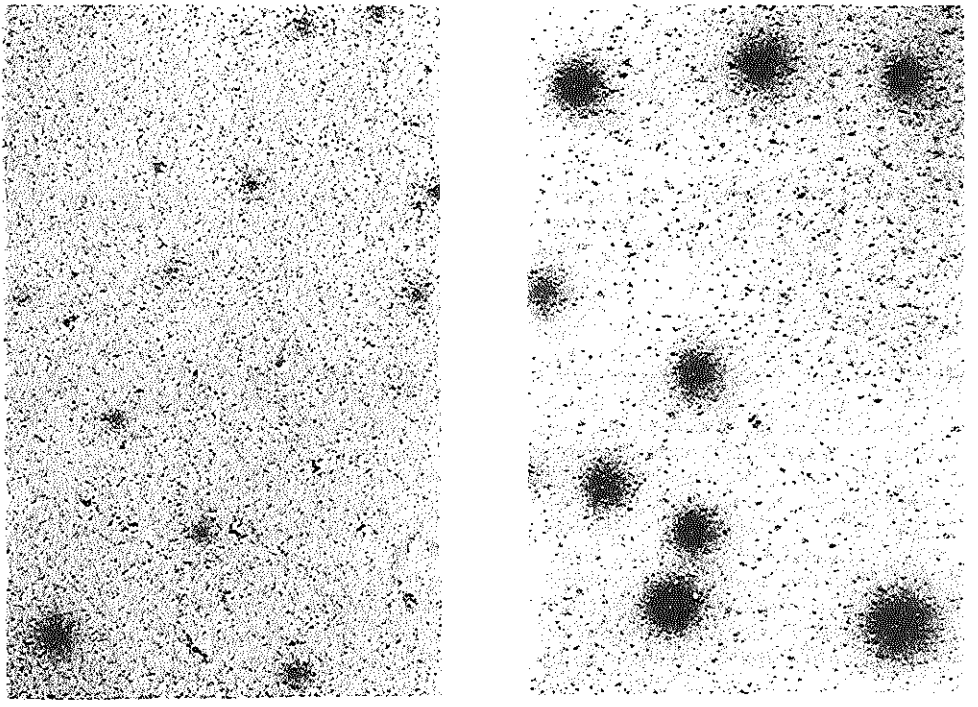

Figure 2. Plaques developed with normal Flow guinea pig serum (left) and with Flow guinea pig serum purified over a Sepharose-protein A column (right). Incubation period 2.5 h. Photograph taken with dark field illumination.

Molinaro and Dray (1974), this problem is avoided by directly coupling the specific antibodies to the target SRBC by $CrCl_3$. The protein A plaque assay, however, is more economical since it requires a smaller amount of rabbit-anti-Ig antibodies. Furthermore, the same batch of protein A-coated SRBC can be used to enumerate Ig-secreting cells in cell suspensions obtained from different species.

Although the data presented in this paper were obtained with the protein A assay in liquid phase and with mouse Ig-secreting cells, it is evident that the purification procedure of the guinea pig complement should also greatly facilitate the more conventional protein A plaque assay in agarose, and the assessment of Ig-secreting cells from species other than mouse.

In conclusion, purification of guinea pig complement over Sepharose-protein A greatly improves the protein A plaque assay described by Gronowicz et al. (1976). This modification reduces the incubation period required, increases the sensitivity of the technique, and facilitates finding an appropriate batch of complement.

ACKNOWLEDGMENTS

We thank Miss Rianne Preesman for technical assistance. Dr. C. Galanos for his kind gift of *S. abortus equi* LPS, Ms. Luciana Forni for the specific rabbit-anti-mouse-IgM antiserum and Ms. Cary Meijerink for typing the manuscript.

This investigation was supported by the Netherlands Foundation for Medical Research (FUNGO) and the Interuniversitary Institute for Irradiation Pathology and Irradiation Protection (IRS).

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CHAPTER V

LOW DOSE X-IRRADIATION OF THYMUS FILLER CELLS IN LIMITING DILUTION CULTURES OF LIPOPOLYSACCHARIDE-REACTIVE B CELLS REDUCES THE BACKGROUND IMMUNOGLOBULIN-SECRETING CELLS WITHOUT AFFECTING GROWTH-SUPPORTING CAPACITY

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In: J. Immunol. Methods 51:323-330 (1982)

ABSTRACT

Frequencies of lipopolysaccharide (LPS)-reactive B cells in the mouse can be determined in the limiting dilution culture system developed by Andersson et al. (1976, 1977a) which is completely dependent upon the presence of thymus filler cells, usually of rat origin. The assessment of B cell clones of mouse origin, however, can be hampered by the occurrence of varying numbers of thymus-derived immunoglobulin (Ig)-secreting cells. The number of these background Ig-secreting cells can be significantly reduced by low dose (110 mgray = 11 rad) X-irradiation of the rat thymus filler cells, without affecting their growth-supporting capacity.

INTRODUCTION

Andersson and co-workers (1976, 1977a) have greatly improved the *in vitro* culture conditions for lipopolysaccharide (LPS)-reactive murine B cells. By adding to the cultures 2-mercaptoethanol, fetal calf serum with low endogenous mitogenicity, and thymus cells, they succeeded in establishing a limiting dilution culture system for LPS-reactive B cells. Under such conditions each LPS-reactive B cell developed into a clone of 15 to about 60 immunoglobulin (Ig)-secreting cells within a 5 day culture period and as a consequence all cultures containing more than 15 Ig-secreting cells are scored as positive (Andersson et al., 1977a, c). We find that under optimal culture conditions, improved by low dose irradiation of rat thymus filler cells as we will show, the average size of clones arising from LPS-reactive B cells ranges from 30 to 70 Ig-secreting cells, whereas the number of irradiated thymocyte cultures with more than 25 background Ig-secreting cells is less than 1 in 32. Thus limiting dilution cultures containing more than 25 Ig-secreting cells are scored as positive.

Originally Andersson et al. (1976, 1977a) used mouse thymocytes. Since as many as 3×10^6 thymocytes/ml were added to the cultures, they had first to be passed through nylon wool to remove contaminating B cells which would have interfered with the limiting dilution analysis of LPS-reactive B cells. Without elimination of the B cells from the thymus filler cells, the number of negative cultures would have been underestimated, resulting in false B cell precursor frequencies.

In a later modification rat thymocytes were used instead (Andersson et al., 1977b, c). This had the advantage that more cells were available and it was also claimed that they did not have to be passed through nylon wool since rat thymus seldom contains LPS-reactive B cells and even if present the latter should not be detected in the protein A plaque assay with antibodies specific for mouse Ig (Andersson et al., 1977b).

However, we have found that varying numbers of rat thymus-derived background Ig-secreting cells appear after 5-7 days of culturing, which sometimes makes proper screening of negative cultures impossible. Nossal and Pike (1978) have also noted that background antibody formation is by no means negligible and Andersson et al. (personal communication) have since encountered the problem of too high background values. It has been reported that nylon wool passaging of thymocytes may reduce their growth-supporting capacity (Nossal and Pike, 1978) and since it is a cumbersome and time-consuming procedure we tried to find an easier method of reducing the number of background Ig-secreting cells.

Irradiation of feeder cells is commonly used to inactivate unwanted properties (Lefkovits and Waldmann, 1979; Reid and Rojkind, 1979), usually with doses as high as 12-50 grays (=1200-5000 rad). Andersson et al. (1977a) irradiated syngeneic thymus filler cells with 15 grays (Gy), but this resulted in a total loss of growth-supporting capacity. The B cell precursor frequencies they found with high-dose irradiated thymus filler cells were as low as in the absence of thymus cells. We report here that X-irradiation of the thymus filler cells with lower doses does not affect feeder capacity, while significantly reducing the number of background Ig-secreting cells. The use of low dose irradiated rat thymocytes allows accurate discrimination between positive and negative cultures, and thereby improves the reliability of the assay.

MATERIALS AND METHODS

Animals

Female C57BL/6J mice, 4-8 weeks old, were purchased from Olac 1976, Blackthorn, England. Female Lewis rats, 4 and 8 weeks old, were purchased from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands.

Cells

The preparation of cell suspensions from thymus and bone marrow (BM) has been described previously (Benner et al., 1981b). During dissection of the thymus, disruption of blood vessels was carefully avoided, and thymus-associated lymph nodes were carefully removed. The cells were washed twice and resuspended in RPMI 1640 medium containing 5% fetal bovine serum (batch T190101S), specifically selected for growth-supporting properties and low mitogenic activity (Gibco Bio-Cult, Irvine, Scotland). Viable cells were counted by trypan blue exclusion.

Irradiation

Thymus cell suspensions were agitated briefly before irradiation at a concentration of 75-150 x 10^6 cells/ml in a Falcon 2057 tube at room temperature with a Philips Müller MG 300 X-ray machine. The conditions of irradiation were 300 kV (constant potential), 10mA, added filtration of 1.0 mm Cu;

focus object distance of 20 cm; and dose rate of 1.16 Gy/min. During irradiation the dose was measured with a NE Ionex 2500/3 dosimeter.

Culture conditions

C57BL/6J BM cells were cultured under limiting dilution conditions together with irradiated or non-irradiated rat thymus cells and 50 µg/ml LPS in 0.2 ml fresh RPMI 1640 medium supplemented as described by Van Oudenaren et al. (1981). Irradiated or non-irradiated rat thymocytes were cultured under the same conditions. The LPS (from *Escherichia coli* 026:B6) was purchased from Difco Laboratories, Detroit, MI. The cultures were assayed for IgM-secreting plaque forming cells (PFC) on day 5 and for IgG2-secreting cells on day 7 of culture. Frequencies of LPS-reactive B cells were calculated, taking the fraction of negative cultures at different cell concentrations as the zero-term of a Poisson distribution (Lefkovits and Waldmann, 1979).

Protein A plaque assay

Ig-secreting cells were assayed by the hemolytic plaque assay as developed by Gronowicz et al. (1976), and modified by Van Oudenaren et al. (1981). The origin, preparation and specificity of the antisera used are described by Benner et al., (1981a).

D37 determination

In radiobiology, D37 values refer to the amount of radiation necessary to reduce by 63% the capacity of a cell population to reproduce indefinitely as calculated from the linear portion of a semi log scale plot (Anderson and Lefkovits, 1980). In our data on rat thymocytes, D37 refers to a reduction in the tested immunologic reactivity to 37% of that for non-irradiated controls, as revealed by total number of PFC or feeder capacity, respectively.

RESULTS

To determine whether PFC were already present in the rat thymus before culture, we tested freshly prepared thymus cell suspensions from 4-week-old and 8-week-old rats for IgM-secreting PFC. This was done with rabbit-anti-mouse-IgM antiserum as developing serum. At both ages only 1-8 PFC were found per 6×10^5 thymocytes. These low numbers can not account for the substantial numbers of IgM-PFC (sometimes up to a few hundred) in 5 day-cultures containing 6×10^5 thymocytes. Similar numbers of PFC in the thymus were found for the other Ig-classes (data not shown).

Since the background thymus-derived Ig-secreting cells in cultures of LPS-activated lymphocytes are not due to persistence of Ig-secreting cells normally present in a rat thymus, the appearance of the Ig-secreting cells in the cultures must be due to activation of B cells from the rat thymus in culture. We therefore investigated whether such activation is due to the *E. coli* LPS or to the endogeneous mitogenic activity of the fetal calf serum used. It appeared that the background thymus-derived IgM-PFC on day 5 were mainly due to the mitogenic activity of the *E.coli* LPS (19.0 ± 1.9 IgM-PFC per 6×10^5 thymocytes cultured in the presence of LPS, as compared to 2.3 ± 1.1 for cultures without LPS). The LPS apparently stimulated a number of rat B cells to differentiate into PFC. Cultures of thymuses from 4-week-old rats developed only 5% of the number of PFC produced by cultures of thymuses from 8-week-old rats, but nevertheless unacceptable numbers of cultures con-

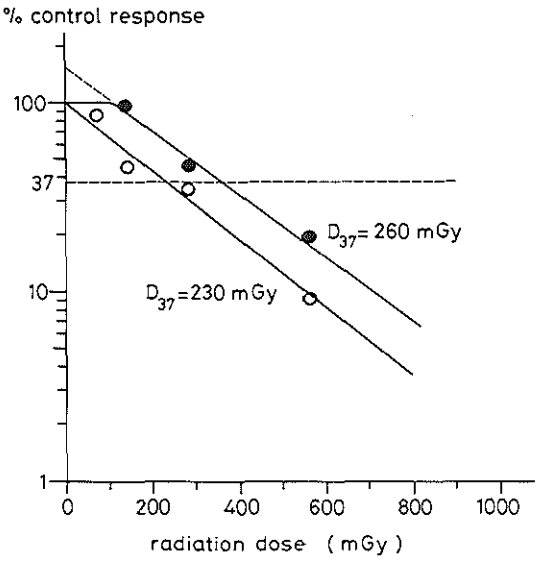

Figure 1. Radiosensitivity of the IgM-PFC response in LPS-activated cultures of rat thymocytes (○) and of the thymocyte growth-supporting capacity in LPS-activated cultures of mouse bone marrow cells (●). 6 x 10^5 irradiated and non-irradiated thymocytes were cultured for 5 days in the presence of E.coli LPS with or without a limiting number of mouse BM cells. Cultures were tested for IgM-PFC by the protein A plaque assay with a rabbit-anti-mouse-IgM antiserum as developing serum. Each closed circle is derived from limiting dilution analysis of the frequency of LPS-reactive BM cells using thymocyte feeder cells, irradiated with the indicated dose. Each frequency analysis is based upon 3 different BM cell inputs (32 cultures/cell input). D37 values were calculated from the linear portion of the curve by the method of least squares. Mean values of 5 (○) and 3 (●) experiments are plotted. The D37 values of the individual experiments were 200, 210, 230, 250 and 270 mGy (○) and 190, 240 and 350 mGy (●), respectively. The reactivities tested are expressed as % of the non-irradiated control reactivity. These control values were 21 ± 4.9 IgM-PFC per culture of 6 x 10^5 thymocytes, and one LPS-reactive B cell per 42 ± 2.2 nucleated murine BM cells.

taining more than 25 PFC were found even with 4-week-old rats.

We next irradiated the thymus cell suspensions with various doses of X-rays, and cultured them together with LPS. Similarly irradiated rat thymocytes were cultured together with mouse BM cells and LPS in order to determine the effect of irradiation upon the thymocyte growth-supporting capacity. Fig. 1 shows that relatively low doses of X-irradiation significantly decreased the number of IgM-PFC in the thymocyte cultures. For thymocytes from 4-week-old rats a D37 of 230 ± 13 mGy was found. The D37 of thymic B cells from 8-week-old rats was 440 ± 44 mGy (5 experiments ranging from 330-580 mGy) indicating that they are less sensitive for irradiation than B lymphocytes

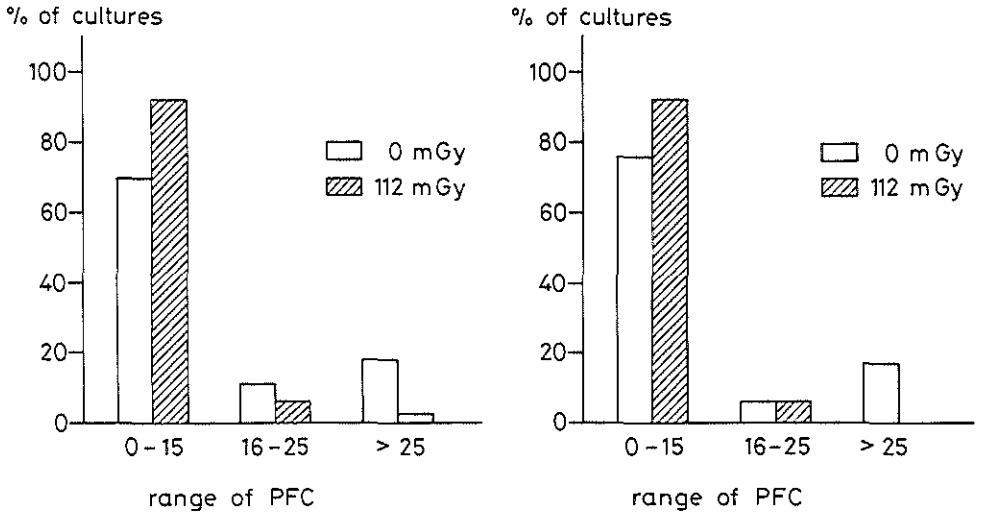

Figure 2. Distribution pattern of the numbers of IgM- (left) and IgG2-PFC (right) in individual cultures of irradiated and non-irradiated thymocytes from 4-week-old rats. Data from 6 (IgM) and 3 (IgG2) individual experiments are pooled. In each experiment 32 cultures of 6 x 10^5 cells were set up, and assayed on day 5 and day 7 by the protein A plaque assay for IgM- and IgG2-PFC, respectively. The IgG2-PFC were assayed with a mixture of rabbit-anti-mouse-Ig antisera specific for IgG2a and IgG2b, respectively.

in the thymus of 4-week-old animals.

The growth-supporting activity of these thymocytes, as tested by BM B cell precursor frequency determinations, was also radiosensitive giving a D37 value of 260 ± 48 mGy. The decrease of IgM-PFC showed an exponential dose-effect relationship, whereas the growth-supporting activity did not significantly decrease in the dose-range of 0-112 mGy, but dropped exponentially with higher doses. The same pattern was found in cultures tested for IgG2-PFC (data not shown). After extrapolation of the exponential portion of the curve to the zero dose an extrapolation number of 152 was found. This figure gives an indication of the width of the shoulder of the dose-response curve at low dose levels.

Comparison of the clone sizes of Ig-secreting cells by BM B cells cultured in the presence of either irradiated (112 mGy) or non-irradiated rat thymocytes revealed no significant differences. However, irradiation with 112 mGy clearly diminished the background number of thymus-derived IgM- and IgG2-PFC per culture. This appears from the distribution pattern of the numbers of IgM- and IgG2-PFC in individual cultures of 112 mGy irradiated and non-irradiated thymocytes (Fig. 2). In the assay for IgM-secretors (Fig. 2, left) 34 out of 192 cultures had more than 25 PFC. This implies at least 6 false positive scorings out of 32 cultures tested per cell input. This number is reduced to a mean of less than 1 after 112 mGy irradiation of the thymus filler cells. In the assay for IgG2 17 out of 96 cultures had more than 25 PFC. However, after irradiation with 112 mGy, none of the cultures had more than 25 PFC (Fig. 2, right).

DISCUSSION

The appearance of rat thymus-derived background Ig-secreting cells in the limiting dilution assay for LPS-reactive murine B cells hampers the use of this assay for accurate B cell frequency determinations. Theoretically, this problem can be mitigated either by purifying the rabbit-anti-mouse-IgM antiserum to eliminate the cross reactivity with rat Ig or by eliminating the unwanted rat B lymphocytes. We chose the latter, since after absorption with rat Ig, rabbit-anti-mouse-Ig anti-sera usually lose their activity for mouse Ig.

Our findings indicate that rat thymus-derived LPS-reactive B cells have a relatively high radiosensitivity (D37 = 230 mGy) which can be used to reduce significantly their numbers in thymus cell suspensions. The low irradiation dose used (112 mGy) does not affect the feeder capacity of the thymocytes, seen in the non-linear part of the curve, referred to as the shoulder (Fig. 1). This is generally thought to reflect the degree of radiation repair that can occur at low irradiation doses, although the possibilities of an effect on regulatory mechanisms and the requirement for several hits for inactivation have also been considered (Anderson and Warner, 1976).

The occurrence of a shoulder in the dose-effect relationship allows the use of low dose X-irradiated rat thymocytes as fillers in the mouse limiting dilution assay for B cells. Without the development of disturbing Ig-secreting cells accurate estimates of mouse B cell precursor frequencies can be made with the protein A plaque assay method. We have shown this especially for the IgM- and IgG2-isotypes, where most interference by unacceptably high numbers of background PFC arises. Frequency determinations of IgG1, IgG3 and IgA are barely hindered by background PFC. Although higher doses of X-irradiation completely eradicate contaminating rat B cells, they also completely abolish the thymocyte feeder capacity (Andersson et al., 1977a).

It is now well established that, in the mouse, B cells generally are more radiosensitive than T cells, and that different subpopulations of B and T cells differ in radiosensitivity (reviewed by Anderson and Warner, 1976). The D37 value of 230 mGy for B cells of 4-week-old rats is low compared with what is reported for mouse lymphocytes. For the latter, values of 700-1450 mGy for *in vitro* irradiated (Anderson and Warner, 1975; Anderson and Lefkovits, 1980) and of 810-940 mGy for *in vivo* irradiated B lymphocytes (Zaalberg et al., 1973) have been determined. The D37 values we find for rat B lymphocytes, however, are comparable with the values of 200 and 220 mGy reported for horse and human lymphocytes, respectively (Dewey and Brannon, 1976; Kwan and Norman, 1977).

A marked difference was found between the D37 values for 4-week-old and 8-week-old rats, the younger being almost twice as sensitive as the older. Since it is thought that the PFC found in the cultured thymocytes of both groups arise from long-lived potentially recirculating B cells (Benner et al., 1977) this difference in radiosensitivity is difficult to explain.

ACKNOWLEDGMENTS

We are indebted to Professor Dr. O. Vos for his continuous support and his critical reading of the manuscript. We thank Mrs. Cary Meijerink-Clerkx for doing the typework.

This investigation was supported by the Interuniversitary Institute for Radiation Pathology and Radiation Protection (IRS), Leiden, The Netherlands, and by the Netherlands Foundation for Medical Research, FUNGO.

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CHAPTER VI

FREQUENCY ANALYSIS OF FUNCTIONAL IMMUNOGLOBULIN C AND V GENE EXPRESSION IN MURINE B CELLS AFTER IN VITRO DIFFERENTIATION OF PRE-B CELLS INTO LIPOPOLYSACCHARIDE-REACTIVE B CELLS

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Submitted for publication

ABSTRACT

During the differentiation of B cells in the murine bone marrow (BM) every B cell becomes committed to produce antibodies of a particular specificity. We investigated whether there is any *in vivo* regulation of the repertoire expression during the differentiation of large pre-B cells into small mature B cells. Therefore, large pre-B cells were separated by 1 g velocity sedimentation and allowed to differentiate *in vitro* into lipopolysaccharide-(LPS) reactive B cells. This population was compared with *in vivo* differentiated small, mature B cells as to the number of IgM-secreting cells they produce after *in vitro* stimulation with LPS, their capacity to switch to the secretion of IgG1 and IgG3 and the absolute frequencies of cells specific for differently haptenated sheep red blood cells (SRBC).

The results presented in this paper indicate that large pre-B cells can equally well differentiate *in vitro* as *in vivo* into LPS-reactive B cells. After *in vitro* stimulation with LPS both populations have an equal ability to differentiate into IgM-secreting cells and to switch to other isotypes. Furthermore, the IgM specificity repertoire of *in vitro* differentiated B cells is not different from that of *in vivo* differentiated B cells. We therefore conclude that the antibody specificity repertoire of B cells and their capacity to switch from IgM to other isotypes can be established independent of *in vivo* regulatory mechanisms.

INTRODUCTION

The generation of antibody diversity is based upon B cell diversification. During ontogeny B cells develop a complete repertoire of antibody specificities, which is continued in the bone marrow (BM) during adult life (1). During the life span, the complete repertoire of antibodies has to be, and is, preserved to respond adequately to the whole diversity of antigenic encounters. It has now been established that the generation of antibody diversity starts with somatic recombinatial events of the germline genes (2,3), but little is known at which stage of differentiation B cells become committed to synthesize a particular specificity. By definition, a B cell becomes immunocompetent at the moment that it can be induced to clonal growth by an antigen. It has been established that before this differentiation stage the cells contain μ-chains in their cytoplasm. Such cells with cytoplasmic μ-

(Cμ)chains and without surface immunoglobulins (sIg) have been defined as pre-B cells (4). Large pre-B cells can be separated from small pre-B cells and small, mature B cells by 1 g velocity sedimentation (5). These large pre-B cells need more time to differentiate into clones of Ig-secreting cells than small, mature B cells (5).

We have separated large pre-B cells from the small BM B cells by 1 g velocity sedimentation and allowed both populations to differentiate *in vitro* into Ig-secreting cells after polyclonal activation by lipopolysaccharide (LPS). Employing a limiting dilution culture system which allows every LPS-reactive B cell to grow and differentiate into a clone of Ig-secreting cells, we compared, at the clonal level, some functional aspects of *in vitro* differentiated B cells with those of normal *in vivo* differentiated B cells. Using the protein A plaque assay and plaque assays specific for differently haptenated sheep red blood cells (SRBC) we determined the frequency of reactive cells as to the isotypes and antibody specificities of the Ig's they can produce.

MATERIALS AND METHODS

Animals

Female C57BL/6J mice, 4- to 8-wk-old, were purchased from Olac Ltd., Bicester, United Kingdom. Female Lewis rats, 4 wk of age, were obtained from the Central Institute for the Breeding of Laboratory Animals, TNO, Zeist, The Netherlands. The mice had been barrier-maintained from birth under specific pathogen-free conditions.

Separation by 1 g velocity sedimentation

BM cell suspensions were prepared as described previously (6) and separated by 1 g velocity sedimentation according to Miller and Phillips (7). Briefly, maximally 1 x 10^8 nucleated cells were suspended in 0.25% bovine serum albumin (BSA) in balanced salt solution (BSS). They were layered on 600 ml of a 1-2% BSA/BSS gradient and allowed to sediment for 4 hours at 4°C. After this period the cells were collected in 10 ml fractions. The total number of nucleated cells per fraction was determined with a Coulter Counter model B (Counter Electronics Ltd., Harpenden, Herts, United Kingdom), while the number of viable nucleated cells was determined by trypan blue exclusion. For mass cultures the cells from the individual fractions 25 to 47 (sedimentation velocity of 3.5 to 7.0 mm/hr) were separately centrifuged, resuspended, diluted and cultured at the appropriate concentration in culture medium. The frequency determinations were done with a pooled 'large cell fraction' enriched for large pre-B cells with a sedimentation velocity of 5.5 to 6.0 mm/hr and compared with a pooled 'small cell fraction' enriched for mature B lymphocytes with a sedimentation velocity of 3.5 to 4.0 mm/hr.

Mass cultures

In mass cultures the BM cell fractions were cultured at 2000 cells per culture with 7.2 x 10^5 irradiated (0.1 Gy) rat thymus cells (8) to support growth and LPS from *Escherichia coli* (026:B6; Difco Laboratories, Detroit, Michigan, USA) at the optimal concentration of 50 μg/ml in 0.2 ml RPMI 1640 medium supplemented with glutamine (4 mM), penicillin (100 U/ml), streptomycin (50 μg/ml), 2-mercaptoethanol (5 x 10^{-5}M), fetal bovine serum (20%) lot 101108 (Sera Lab Ltd., Sussex, United Kingdom), specifically selected for growth supporting capacity and low endogenous mitogenic activity. In the

kinetic experiments, cultures were assayed with the protein A plaque assay from day 4 to day 6 for IgM-secreting cells and from day 5 to 7 for IgG1-secreting cells.

Frequency determinations
Frequencies of mitogen-reactive B cells secreting IgM-, IgG1- and IgG3-isotypes or IgM-antibodies specific for differently haptenated SRBC, were estimated by the method originally described by Andersson et al. (9). Varying numbers of BM cells of different fractions were cultured under culture conditions as described above. For each cell concentration 32 replicate cultures were set up, routinely. Control cultures contained rat thymus cells, but not mouse lymphoid cells. The cultures were assayed for total IgM plaque-forming cells (PFC) from day 5 to day 7. Total IgG1- and IgG3-PFC were assayed from day 6 until day 8. The IgM-PFC specific for differently haptenated SRBC were assayed on the day that the IgM-secreting clones were most frequent which was day 5 and day 7 for the small and the large cell fractions, respectively. The maximum number of PFC observed in the control cultures was 25 for IgM-PFC and 10 for the other isotypes. Control cultures assayed with the haptenated SRBC never contained more than five antigen-specific PFC. In the limiting dilution assay, cultures were scored as positive when they yielded more than 10 PFC above the maximum number of PFC found in the control cultures. The frequency of reactive cells was determined by confirming the fraction of negative cultures to the zero term of the Poisson distribution.

Plaque assays for Ig- and antibody-secreting cells
The target cells for the protein A plaque assay and the antigen-specific plaque assay were prepared and the plaque assays were performed as has been extensively described (10). For the antigen-specific plaque assays, 5-iodo-3-nitrophenyl (NIP), 4-hydroxy-3,5-dinitrophenyl (NNP) and 2,4,6-trinitrophenyl (TNP) were coupled to SRBC with 4, 2 and 30 mg of the hapten per ml of washed packed SRBC, respectively. They are referred to as NIP_4-, NNP_2- and TNP_{30}-SRBC, respectively.

Immunofluorescence
Cell suspensions were stained for sIg by incubation of cells with appropriate dilutions of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (Fc) serum (batch nr. 35-178, Nordic, Tilburg, The Netherlands). 1×10^6 cells in 100µl of 1% BSA in phosphate buffered saline (PBS) (pH 7.8) were stained with 15µl of a 1 to 10 dilution of the antiserum in PBS for 30 min on melting ice. Afterwards the cells were washed twice with 1% BSA in PBS and finally mounted in glycerol/PBS (9:1) with 1 mg/ml phenylenediamine (pH 8.0) to prevent fading of the fluorescence (11). For double staining of cytoplasmic Ig positive and surface Ig negative ($C\mu^+/sIg^-$) cells, cytocentrifuge slides were made after membrane staining. These slides were fixed for 30 min in 5% acetic acid in absolute alcohol and, after washing with PBS, stained with 30µl of tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgM (Fc) serum (Batch no. 36-378, Nordic, Tilburg, The Netherlands). After washing with PBS, the preparations were left overnight in PBS at 4°C to minimize the background fluorescence. The slides were mounted as described before and were scored both for $C\mu^+/sIg^-$ cells and for sIg^+ cells. Fluorescence microscopy was performed with a Zeiss standard microscope equipped with a Zeiss IV/F epi-illuminator, an Osram HBO 50 mercury lamp and filter combinations for the selective visualization of FITC and TRITC. The percentages positive cells were calculated as the quotient of the fluores-

cent positive cells to the total of nucleated cells on the phase-contrast image. At least 200 cells were counted per sample.

RESULTS

Analysis of the velocity sedimentation profile
BM cells were separated by 1 g velocity sedimentation. The profile of the number of nucleated cells per fraction is shown in Figure 1. A number of 2000 nucleated cells of each fraction was cultured with LPS to analyze the growth kinetics of the B cells. As can be seen in Figure 1, the fractions containing large cells as well as the fractions containing small cells gave a significant IgM-response on day 4, with the most prominent response at 4.7 mm/hr. However, on day 6 the response in the fractions containing small cells was reduced about 6-fold while the response of the fractions containing large cells was at least the same or even greater than on day 4. This shows that the large-cell fractions contain a subpopulation of cells that gives a delayed response as compared with the small cells.

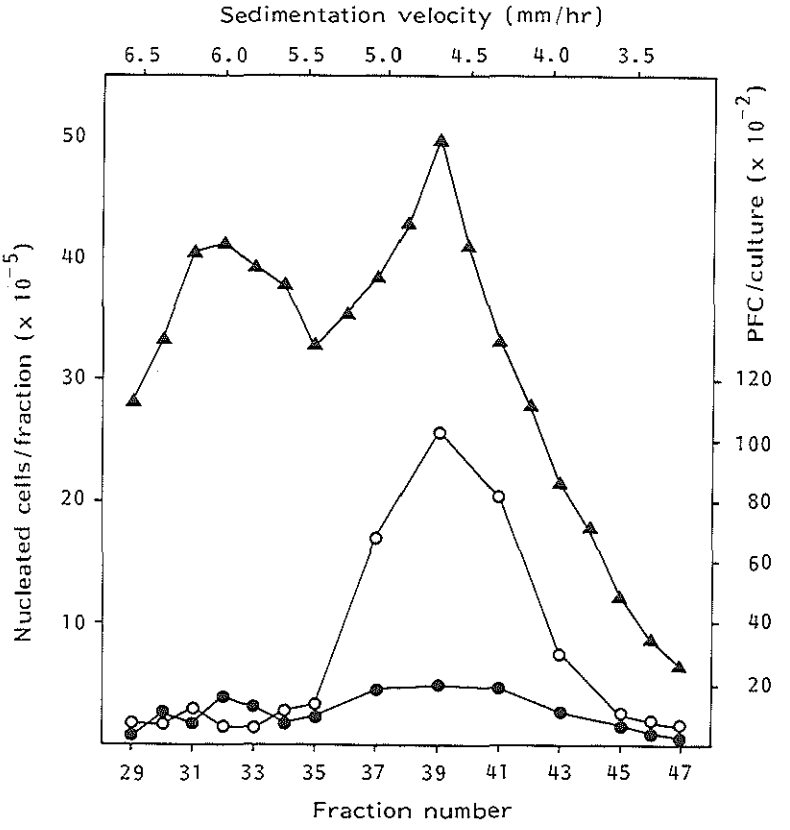

Figure 1. The number of nucleated cells (▲) per fraction as determined by Coulter Counter analysis. The number of IgM-PFC per culture was assayed on day 4 (○) and day 6 (●). Each culture contained 2000 viable nucleated cells.

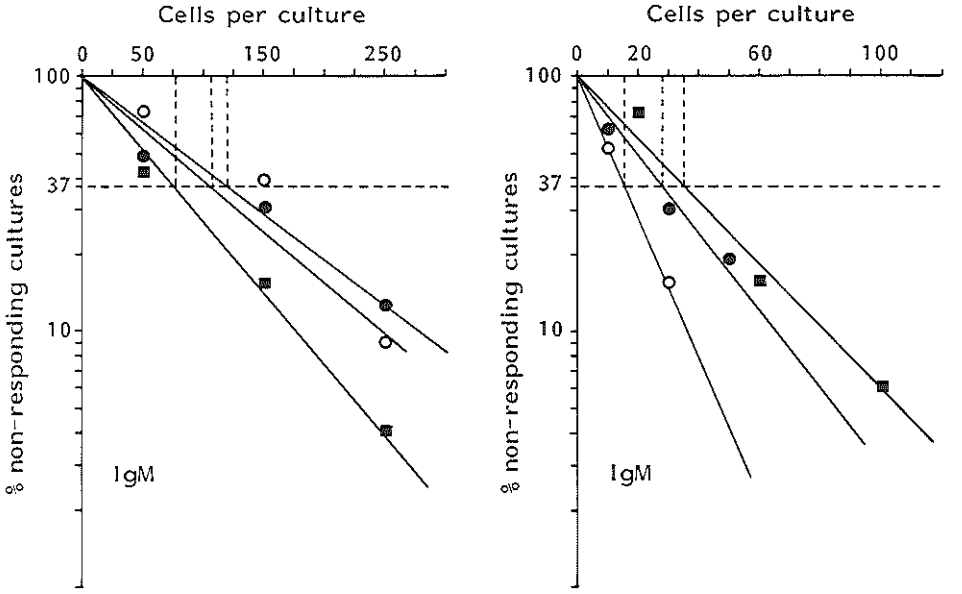

Figure 2. Limiting dilution analyses of LPS-reactive B cells among C57BL/6J bone marrow cells separated by 1 g velocity sedimentation in a large- (pre-B) cell (left) and a small- (B) cell fraction (right). Clones of IgM-secreting cells were determined on day 5 (○), day 6 (●) and day 7 (■).

Frequencies of LPS-reactive B cells in small- and large-cell fractions
To avoid both small and large cells contaminating each other, we pooled the cells with a sedimentation velocity of 3.5 to 4.0 mm/hr ('small-cell fraction') as well as the cells of 5.5 to 6.0 mm/hr ('large-cell fraction'). Cells from both fractions were cultured with LPS under limiting dilution culture conditions. On day 5, 6 and 7, the cultures were analyzed for the presence of IgM-secreting cells. Subsequently, the frequency of LPS-reactive B cells in each fraction was calculated. As can be seen in Figure 2, the highest frequency of LPS-reactive B cells was found for the small cells on day 5 and for the large cells on day 7. Since the maximum response in the small-cell fraction ('small B cells') was found on day 5, the delayed response by the fraction of large cells indicates enrichment for large pre-B cells, which require two days to differentiate into LPS-reactive B cells (5), and then another five days to give rise to clones of IgM-secreting cells (5).

Number of pre-B cells and B cells in small- and large-cell fractions
The percentages of sIg^+ positive and $C\mu^+/s\mu^-$ cells were determined in unseparated BM and in the large- and small-cell BM fractions by immunofluorescence staining. In the small-cell fraction 18.5% sIg^+ cells was found and in the large-cell fraction 1.7% sIg^+ cells (Table 1). The occurrence of LPS-induced IgM-secreting clones in the large-cell fraction assayed on day 5 (Table 2) must be due to this 1.7% of contaminating B cells that occurs in this fraction. Assuming that the contaminating LPS-reactive B cells within

Table 1

Immunofluorescence staining of unseparated BM cells and BM cells separated by 1 g velocity sedimentation

	% sIg^+ [1] B cells	% $C\mu^+/S\mu^-$ [2] pre-B cells
Bone marrow	7.5 ± 2.5	5.5 ± 2.1
Large-cell fraction (5.5-6.0 mm/hr)	1.7 ± 0.4	5.1 ± 1.9
Small-cell fraction (3.5-4.0 mm/hr)	18.5 ± 3.1	7.4 ± 2.0

1. Percentage of sIg^+ cells as found by membrane immunofluorescence staining with anti-Ig serum.
2. Percentage of $C\mu^+/S\mu^-$ cells as found by membrane and cytoplasmic double staining with anti-μ serum.

the large-cell fraction have the same growth kinetics as the LPS-reactive B cells in the small-cell fraction (Table 2), the incidence of LPS-induced IgM-secreting clones on day 7 after culture of the large-cell fraction should have decreased to the same extent as in the small-cell fraction. In the latter fraction the incidence decreased from 1 in 16 to 1 in 54, thus by a factor of 3.4. If the day 7 response by the large-cell fraction was completely due to contamination with LPS-reactive B cells, the incidence of IgM-secreting clones should have decreased from 1 in 154 to 1 in 524. On the contrary, the incidence of IgM-secreting clones increased from 1 in 154 to 1 in 99 when assaying on days 5, 6 and 7, indicating that pre-B cells occurred in the large-cell fraction. Thus, among the IgM-secreting clones from the large-cell fraction on day 7, a maximum of (99/524 x 100=) 19% may have resulted from contaminating B cells. Thus the IgM response by the large-cell fraction is for at least 81% caused by pre-B cells that have differentiated *in vitro* into LPS-reactive B cells.

Switch frequencies of in vitro differentiated B cells

In vitro differentiated B cells can switch from IgM to IgG1 and IgG3 (Table 2). By dividing the frequency of IgG1- and IgG3-secreting clones by the frequency of IgM-secreting clones, the relative switch frequency can be calculated. In the large-cell fraction and the small-cell fraction 7 and 6% of the B cells switched to IgG1, and 12 and 21% switched to IgG3, respectively (Table 2). This indicates that both populations have roughly the same proportion of cells that can switch from IgM to another isotype.

Frequencies of antigen-specific in vitro differentiated B cells

The frequency of a number of antibody-specificities was determined among the LPS-induced IgM-secreting clones arising from the large-(pre-B) and small-(B) cell fractions. This was done in plaque assays specific for NIP_4-, NNP_2- and TNP_{30}-SRBC. By dividing the frequency of the specific B cells by the frequency of all LPS-reactive B cells the relative frequency of antigen-specific IgM-secreting clones was calculated. It was found that in the large-cell fraction 1 in 15, 1 in 17 and 1 in 99 IgM-secreting clones were reactive

Table 2

Frequencies of LPS-reactive B cell clones secreting IgM, IgG1 and IgG3 after *in vitro* differentiation of pre-B cells into LPS-reactive B cells

Ig isotype	pre B cell fraction[1]					B cell fraction				
	day 5	day 6	day 7	day 8	% switch	day 5	day 6	day 7	day 8	% switch
IgM	1 in 154[2] (121-193)	1 in 123 (93-167)	1 in 99 (68-136)	-	-	1 in 16 (11-21)	1 in 27 (14-33)	1 in 54 (27-86)	-	-
IgG1	-	1 in2816 (2033-3503)	1 in1942 (1812-2048)	1 in 1356 (854-1977)	7[3]	-	1 in 439 (256-717)	1 in 273 (263-283)	1 in 530 (399-588)	6
IgG3	-	1 in1607 (1264-1949)	1 in1455 (848-2062)	1 in 848 (725-970)	12	-	1 in 105 (94-114)	1 in 76 (43-118)	1 in 138 (125-149)	21

1. Bone marrow cells were separated by 1 g velocity sedimentation in a large pre-B cell fraction (5.5-6.0 mm/hr) and a small B cell fraction (3.5-4.0 mm/hr).
2. Frequencies were calculated as fraction of all viable nucleated cells. Figures represent the arithmetic mean of the data from 3 or 4 separate experiments. The upper and lower values of these experiments are given in parentheses.
3. Switching percentages were calculated as the quotient of the frequency of clones secreting IgG1 or IgG3 (day 8 for the pre-B cell fraction and day 7 for the B cell fraction) and the frequency of clones secreting IgM (day 7 for the pre-B cell fraction and day 5 for the B cell fraction).

against NIP_4-SRBC, NNP_2-SRBC and TNP_{30}-SRBC, respectively (Table 3). Among the *in vivo* differentiated B cells (small-cell fraction) 1 in 21, 1 in 19 and 1 in 104 reactive B cells were specific for these antigens, respectively. Apparently, the antibody-specificity repertoire as determined with these antigens is essentially the same in both fractions.

Table 3

Frequencies of B cell clones secreting antibodies to NIP_4-SRBC, NNP_2-SRBC and TNP_{30}-SRBC after *in vitro* differentiation of pre-B cells into LPS-reactive B cells

Antigen	pre-B cell fraction[1]	B cell fraction
NIP_4-SRBC	1 in 15[2] (10,13,23)	1 in 21 (17,24)
NNP_2-SRBC	1 in 17 (10,15,25)	1 in 19 (18,18,22)
TNP_{30}-SRBC	1 in 99 (46,72,178)	1 in 104 (96,101,117)

1. Bone marrow cells were separated by 1 g velocity sedimentation in a large pre-B cell fraction (5.5.-6.0 mm/hr) and a small B cell fraction (3.5-4.0 mm/hr).
2. Frequencies of antigen-specific B cells were calculated as a fraction of all LPS-reactive B cells which was 1 in 99 and 1 in 16 among the differentiated progeny of the pre-B cell fraction and the B cell fraction, respectively. The figures represent the arithmetic mean of 2 or 3 individual experiments. The data of the individual experiments are given in parentheses.

DISCUSSION

The percentages of $C\mu^+/sIg^-$ cells and sIg^+ cells found in the present study employing normal BM of C57BL/6J mice are in agreement with data from others concerning C57BL/6 and CBA mice (12,13). In the culture system used, cells with pre-B cell characteristics are thought to have differentiated *in vitro* into Ig-secreting clones. However, the large cells that we selected as pre-B cells represent about one third of the total BM pre-B cell pool (12). The latter also includes a more mature non-dividing population of small pre-B cells, likely to be derived from the rapidly cycling large ones (12-15).

Andersson et al. have shown that LPS polyclonally activates one third of all B cells to proliferate and differentiate into a clone of Ig-secreting cells, independent of their V-region specificity (9). These LPS-reactive B cells constitute a representative fraction of the B lymphocyte compartment with regard to the rate of decay and recovery, as has been shown by *in vivo* killing of cells in S-phase by hydroxyurea (HU) (16,17). However, there is some evidence that the fraction of precursors specific for the monoclonal Ig 174 to β-galactosidase is not randomly distributed among B cell subpopulations activated by different mitogens (18).

The cell separation procedure used has the advantage of separating the cells only on the base of their size, which leaves them in an as optimal as possible physiological condition. Employing the same procedure, Lau et al. also described a delayed IgM response in the large-cell fraction (5) accompanied by the same increase of the frequency of LPS-induced clones of IgM-secreting cells as we observed. Our results also confirm an earlier report, which suggests that day 5 is the optimal day for detecting IgM-secreting clones originating from small LPS-reactive B cells (19).

The data presented in this paper show that the *in vitro* differentiated B cells can switch from the secretion of IgM to the secretion of other isotypes (Table 2). From the *in vitro* and the *in vivo* differentiated B cells the same percentage switched to IgG1 secretion after stimulation with LPS, while somewhat less cells among the *in vitro* differentiated B cells switched to IgG3 secretion. The switch percentages found in the B cell fraction are in good agreement with earlier findings (20). Several reports have shown that the switch percentages from IgM to other isotypes can be influenced by the nature of the activating signal (21-24). With helper T cells a higher proportion of the cells could be induced to switch to IgG1-secretion, while after stimulation with LPS a preferential switch to IgG3-secretion was found (22, 24). At present we do not know whether the difference we found in switch frequency to IgG3 is due to a different regulation of the isotype switch in both B cell populations.

The antibody specificities of the *in vitro* differentiated B cells were examined in plaque assays using differently haptenated SRBC and compared with *in vivo* differentiated LPS-reactive B cells. We found a striking correlation between the frequencies of IgM-secreting cells that were specific for NIP_4-, NNP_2-, and TNP_{30}-SRBC among the two cell populations (Table 3) and these frequencies agree well with previous data from LPS-reactive B cells in normal BM (10). Nishikawa et al. have recently found comparable results for the expression of the Ac38 and Ac146 idiotypes of antibodies against the hapten NP (4-hydroxy-3-nitrophenyl) (25). They selected pre-B cells by eliminating the mature B cells by adherence to anti-Ig coated plates. They did not find any difference between *in vitro* and *in vivo* differentiated LPS-reactive B cells with regard to the occurrence of the Ac38 and Ac146 idiotypes, which are much less frequent than the antibody specificities analyzed by us. Similar results were obtained by Juy et al. for precursor cells synthesizing Ig which reacts with the monoclonal anti-M460 antibody F6(51) (26). So in a large spectrum of specificities, ranging from very frequently occurring to seldomly occurring ones, the same frequencies of specificities were found for *in vitro* and *in vivo* differentiated LPS-reactive B cells, indicating that there is no selection for V-region specificity during the maturation of large pre-B cells into LPS-reactive small B cells under the conditions employed.

Klinman and coworkers (27,28) studied the mouse B cell repertoire using an *in vitro* splenic focus assay. They compared the repertoire expressed by splenic B cells with that expressed by BM derived sIg^- B cell precursors and concluded that in both populations the responses to 2,4-dinitrophenyl (DNP), influenza agglutinin (HA) and phosphorylcholine (PC) are comparable in diversity, which is in accordance with the aforementioned data. As we studied specificities of IgM-producing cells originating from newly-formed B cells (16,17), it is likely that we studied the germ-line repertoire only. Therefore, our results can not be related to the overall antibody-specificity repertoire represented by the 'spontaneously' occurring ('background') Ig-

secreting cells. The latter is different from the repertoire of newly-formed B cells, which is the same under various conditions (10,29). The repertoire of the background Ig-secreting cells, however, is different under different conditions, as has been shown by the analysis of the specificity repertoire of background IgM-secreting cells in BM and spleen of young and old athymic nude (Hooijkaas et al., submitted for publication) and thymus-bearing mice (10), suggesting *in vivo* regulatory mechanisms in the expression of the mature B cell repertoire (28), which also includes B memory cells and B cells expressing other isotypes than IgM.

One might speculate that the presumptive selection of the IgM antibody-specificity repertoire takes place either at a very early stage of the B cell differentiation or at the level of the more mature (long-lived) B cells, that are not reactive to LPS. These two compartments of the B cell differentiation lineage were not analyzed in our system which detects only differentiation stages ranging from the large pre-B cell to the newly-formed LPS-reactive B lymphocyte.

ACKNOWLEDGMENT

We gratefully acknowledge Miss K. Benne for technical assistance and Mrs. C.J.M. Meijerink-Clerkx for typing the manuscript.

This investigation was supported by the Netherlands Foundation for Medical Research (FUNGO).

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CHAPTER VII

IMMUNOGLOBULIN ISOTYPE EXPRESSION
II. FREQUENCY ANALYSIS IN MITOGEN-REACTIVE B CELLS

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In: Eur. J. Immunol. 11:799-804 (1981)

SUMMARY

The frequency of lipopolysaccharide (LPS)-reactive B cells developing into clones that secrete various immunoglobulin (Ig) classes has been determined *in vitro*, in cells from BALB/c mice, under culture conditions which detect all growth-inducible cells. Secretion of the different Ig classes was assessed in the protein A plaque assay for Ig-secreting, plaque-forming cells by using developing antisera specific for either IgM, IgG1, IgG2a, IgG2b, IgG3 or IgA. In all lymphoid organs tested (spleen, bone marrow, mesenteric lymph nodes and thoracic duct), a considerable proportion of all B cells (5-20%) was induced by LPS to yield a clone of IgM-secreting cells. Frequency determinations of LPS-reactive cells giving rise to descendants secreting other Ig isotypes revealed that, on an average, and irrespective of the origin of the cells, 7% of all IgM-secreting clones switched to the synthesis of IgG1, 39% to IgG2, 41% to IgG3 and 1% to IgA. Roughly the same frequencies of B cells switching C_H gene expression were found among spleen cells of athymic nude mice. No correlation was found between the clonal frequencies of C_H gene expression in polyclonally activated B cells and the *in vivo* 'background' Ig-secreting cells suggesting that the C_H gene expression in B cells is influenced by the quality of stimulation and other regulating influences.

1. INTRODUCTION

Immunoglobulins (Ig) are heterogeneous with respect to the variable region repertoire as well as the heavy and light chain isotype distribution. One of the puzzling questions concerning the structure and organization of Ig genes is related to the relationship between those two types of heterogeneity, and has been long known as the 'switch' in Ig class. V gene expression in B lineage cells is 'fixed', *i.e.* a given B lymphocyte and all its progeny express the same V_HV_L pair, and all cells of a clone are, therefore, committed to make antibodies of identical specificity (1). In contrast, heavy chain C gene expression varies among the progeny of a single activated B lymphocyte (2,3).

At present, little is known about the variation of C gene expression in single clones of activated B cells and its regulation. *In vivo* experiments and stimulation of mass cultures of lymphoid cells seem to be less appropriate to investigate these questions, since they are difficult to interpret in clonal terms. The recent development of *in vitro* techniques which permit the activation and growth of single B lymphocytes under limiting dilution conditions (4), and the availability of the protein A plaque assay for detection of all cells secreting Ig of a certain isotype (5) permit the analysis of the frequency of C gene expression at the clonal level.

We sought to obtain quantitative estimates of the various 'switch frequencies' in large populations of B lymphocytes, in the absence of regulatory influences mediated by T lymphocytes and independently of Ig receptor recognition of antigen. We have employed these techniques for investigating the frequency of Ig C_H gene expression in lipopolysaccharide (LPS)-reactive B cells from murine spleen, bone marrow (BM) and mesenteric lymph nodes (MLN), and among thoracic duct lymphocytes (TDL). The results show that a relatively constant proportion of all IgM-secreting clones switches to the synthesis of IgG1, IgG2, IgG3 and IgA. This was found for B cells from all lymphoid organs tested, and regardless of whether they were obtained from thymus-bearing mice or from athymic nude mice. This stands in clear contrast to the organ-specific Ig class distribution profile of *in vivo* background Ig-secreting cells.

2. *MATERIALS AND METHODS*

2.1 *Animals*

Female BALB/c nude and normal mice, 8-10 weeks of age, were purchased from the Radiobiological Institute TNO, Rijswijk, NL, and Gl. Bomholtgård, Ry, Denmark. Lewis male rats, 4 weeks of age, were obtained from the Institut für Biologisch-Medizinische Forschung AG, Füllinsdorf, Switzerland, and the Central Institute for the Breeding of Laboratory Animals, TNO, Zeist, NL. The mice were barrier-maintained under specific pathogen-free conditions, in cages of five.

2.2 *Mitogen*

Salmonella abortus equi LPS was kindly provided by Drs. C. Galanos and O. Lüderitz, Max-Planck-Institut für Immunbiologie, Freiburg i. Br., FRG. It was used in cultures at an optimal concentration of 50 µg/ml.

2.3 *Cells*

Thymus, spleen, BM and MLN cell suspensions were prepared by passing the cells through a nylon gauze filter with 100 µm openings, as described previously (6). TDL were collected by overnight drainage of the thoracic duct according to the procedure described by Sprent (7). Viable cells were counted by the trypan blue exclusion method. Total nucleated cells were counted with a Coulter Counter model B (Coulter Electronics Ltd., Harpenden, Herts., GB).

2.4 *Frequency determinations*

Absolute B cell frequencies were estimated by the method of Andersson et al. (4). Varying numbers of lymphoid cells were cultured in Microtest II (Falcon Plastics, Oxnard, CA, No. 3040) tissue culture plates together with 6×10^5 growth-supporting rat thymus cells and 50 µg/ml LPS in 0.2 ml of

RPMI 1640 medium supplemented with L-glutamine (4 mM), penicillin (100 IU/ml) and streptomycin (50 µg/ml), 2-mercaptoethanol (5×10^{-5} M) and fetal bovine serum (20%), batch U781402N (Gibco Biocult., Irvine, Scotland), specifically tested for growth-supporting properties and low endogenous mitogenic activity. Routinely, 32-36 replicate cultures were set up for each cell concentration and, as controls, containing no mouse lymphoid cells. The cultures were assayed on day 5 or 7 for IgM- or IgG- and IgA-secreting plaque-forming cells (PFC), respectively. In the absence of added spleen cells, a maximum of 5 to 25 PFC was found per well, depending on the Ig (sub)class. Cultures were scored as positive when they yielded at least 10 PFC more than the maximum number of PFC in control 'filler cell' cultures.

2.5 Plaque assays for Ig-secreting cells

IgM-, IgG1-, IgG2-, IgG3- and IgA-secreting cells were assayed by a modified hemolytic plaque assay employing *Staphylococcus aureus* protein A (Pharmacia, Uppsala, Sweden)-coated sheep erythrocytes (SRBC) and Ig class- and subclass-specific rabbit anti-mouse Ig antibodies as developing antibodies, in the presence of guinea pig complement (Behringwerke, Marburg/Lahn, FRG). This assay has been developed by Gronowicz et al. (5). We employed the assay with some minor modifications as described previously (8). The origin and/or preparation and purification of the rabbit anti-mouse Ig antisera have also been described previously (8). Before use, the protein A-binding fraction of antibodies was isolated on a Sepharose-protein A column (Pharmacia) according to Goding (9). The specificity of all antisera was confirmed in protein A plaque assays with the appropriate myeloma cell suspensions.

3. RESULTS

3.1 Frequencies of LPS-reactive B cells in various lymphoid organs developing to clones of IgM-, IgG1-, IgG2-, IgG3 and IgA-secreting PFC

We have employed culture conditions which allow every growth-inducible B cell to grow and mature into a clone of Ig-secreting cells upon stimulation by LPS (4). By reducing the numbers of spleen cells added to each culture, reactive B cells become limiting, and fluctuating conditions are reached in which a fraction of all cultures is negative. The fraction of negative cultures for the various cell concentrations was conformed to the zero-term of the Poisson distribution. Therefore, frequencies of reactive cells could be determined at cell concentrations yielding, on an average, 1 clone/culture. The class or subclass of the secreted Ig was determined by using developing antisera specific for either IgM, IgG1, IgG2a+IgG2b, IgG3 or IgA, in the protein A plaque assay.

Frequencies of LPS-reactive B cells developing to clones of IgM-, IgG1-, IgG2-, IgG3- and IgA-secreting cells were determined in spleen, BM, MLN and among TDL of young adult BALB/c mice, and in spleen of age-matched athymic BALB/c nude mice. Fig. 1 shows the results of such limiting dilution analyses of B cells from the spleen of BALB/c and BALB/c nude mice. Spleen cells from euthymic mice as well as from athymic mice give rise to clones of Ig-secreting cells of all heavy chain isotypes tested. For all isotypes, frequencies were higher for 'nude' than for normal spleen cells (Fig. 1). However, after correction for the B cell content, which is about 65% in nude spleen and 45% in normal spleen, the frequencies of LPS-inducible B cells yielding clones of IgM-, IgG1-, IgG2-, IgG3- and IgA-secreting cells were about the same among spleen cells from both sources (Table 1). Most frequent were clones of

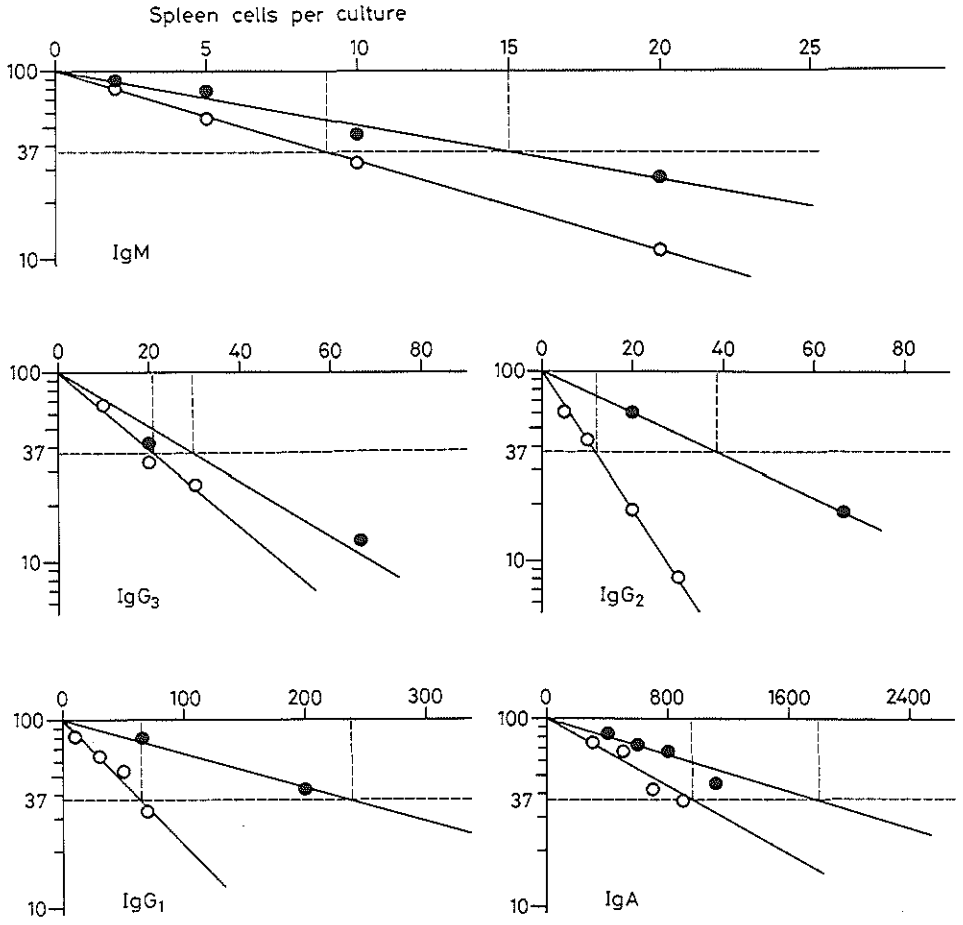

Figure 1. Limiting dilution analysis of LPS-inducible B cells in the spleen of athymic BALB/c nude mice (○) and normal BALB/c mice (●). Cultures were assayed for IgM-secreting PFC on day 5 of culture, while IgG1-, IgG2-, IgG3- and IgA-secreting cells were assayed on day 7 of culture. The mean clone sizes were calculated to range from 30 to 70 PFC, for the different Ig (sub)-classes and day of assay.

IgM-secreting cells, followed by clones of IgG3-, IgG2-, IgG1- and IgA-secreting cells in order of decreasing frequency.

We have previously demonstrated, by probability analysis of limiting dilution experiments (3), that all IgG1- and IgG2a-secreting cells in LPS-stimulated cultures develop in B cell clones that previously secreted IgM. The evidence was obtained under such conditions that 90% of all cultures contained a single clonal precursor, and it was concluded from these findings that IgG-secreting cells only appeared in cultures containing IgM-

Table 1

Frequencies of LPS-reactive B cells in the spleen of athymic nude mice and in spleen and BM of normal thymus-bearing mice, and the heavy chain isotype of the Ig secreted by their progeny

Ig isotype secreted by PFC	BALB/c nude spleen			BALB/c spleen			BALB/c BM		
	Absolute frequency[a]	B cells activated by LPS[b]	Switching frequency[c]	Absolute frequency	B cells activated by LPS	Switching frequency	Absolute frequency	B cells activated by LPS	Switching frequency
IgM	1 in 8 (6;9)	1 in 5	-	1 in 14 (12;15)	1 in 6	-	1 in 100 (90;100;100)	1 in 6	-
IgG1	1 in 78 (65;90)	1 in 52	1 in 10 (10%)	1 in 180 (120;240)	1 in 81	1 in 13 (8%)	1 in 1900 (1900)	1 in 114	1 in 19 (5%)
IgG2[d]	1 in 15 (12;18)	1 in 10	1 in 1.9 (52%)	1 in 35 (32;38)	1 in 16	1 in 2.5 (39%)	1 in 230 (150;200;350)	1 in 14	1 in 2.3 (43%)
IgG3	1 in 20 (20;21)	1 in 13	1 in 2.5 (40%)	1 in 30 (30;30)	1 in 14	1 in 2.2 (45%)	1 in 320 (160;340;460)	1 in 19	1 in 3.2 (31%)
IgA	1 in 665 (430;900)	1 in 432	1 in 89 (1.1%)	1 in 1325 (850;1800)	1 in 596	1 in 95 (1.1%)	1 in 6500 (6500)	1 in 390	1 in 65 (1.5%)

a. Calculated as fraction of all viable nucleated cells. Figures represent the arithmetic mean of the data from different experiments. The figures from the individual experiments are given in parentheses.

b. Assuming that in BALB/c, 65% of all nucleated nude spleen cells, 45% of all nucleated normal spleen cells and 6% of all nucleated BM cells are B cells (L. Forni, personal communication).

c. Calculated as the fraction of IgM-secreting cells that has switched to the synthesis of another Ig heavy chain isotype.

d. The total of IgG2a- and IgG2b-secreting PFC was determined by using a mixture of anti-IgG2a and anti-IgG2b antibodies in the plaque assay.

Table 2

Frequencies of LPS-reactive B cells in MLN and among TDL and the heavy chain isotype of the Ig secreted by their progeny

Ig isotype secreted by PFC	MLN Absolute frequency[a]	MLN B cells activated by LPS[b]	MLN Switching[c] frequency	TDL Absolute frequency	TDL B cells activated by LPS	TDL Switching frequency
IgM	1 in 34 (12;30;60)	1 in 14	-	1 in 70 (60;80)	1 in 18	-
IgG1	1 in 590 (460;600;700)	1 in 236	1 in 17 (6%)	1 in 1800 (1800)	1 in 450	1 in 26 (3.9%)
IgG2[d]	1 in 120 (52;57;260)	1 in 48	1 in 3.5 (28%)	1 in 205 (160;250)	1 in 51	1 in 2.9 (34%)
IgG3	1 in 70 (57;80)	1 in 28	1 in 2.1 (49%)	1 in 175 (100;250)	1 in 44	1 in 2.5 (40%)
IgA	1 in 2500 (2500)	1 in 1000	1 in 74 (1.4%)	1 in 6200 (6200)	1 in 1550	1 in 89 (1.1%)

a. Calculated as fraction of all viable nucleated cells. Figures represent the arithmetic mean of the data from different experiments. The figures from the individual experiments are given in parentheses.
b. Assuming that in BALB/c, 40% of all MLN and 25% of all TDL are B cells (L. Forni, personal communication).
c. Calculated as the fraction of IgM-secreting cells that has switched to the synthesis of another Ig heavy chain isotype.
d. The total of IgG2a- and IgG2b-secreting PFC was determined by using a mixture of anti-IgG2a and anti-IgG2b antibodies in the plaque assay.

secreting cells. Furthermore, clones of IgM-secreting cells were most frequent and largest after a 5-day culture period, whereas clones of IgG1- and IgG2a-secreting cells were hardly detectable by day 5, and most frequent and largest by day 7 (3). Recent experiments indicate that both criteria also hold for clones of IgG2b-, IgG3- and IgA-secreting cells (data not shown) suggesting that such clones, like IgG1- and IgG2a-secreting cells, develop within clones that initially secrete IgM. Thus, the relative 'switch frequency' for each of the various heavy chain isotypes can be calculated by dividing the absolute frequency for that isotype by the absolute frequency for IgM. In the various organs tested, the 'switch frequencies' were, on an average, 0.07 for IgG1, 0.39 for IgG2, 0.41 for IgG3 and 0.01 for IgA (Tables 1 and 2).

Frequency analyses of LPS-inducible B cells in BM, MLN and among TDL yielded smaller absolute frequencies of reactive cells, but similar relative 'switch frequencies' for the various isotypes as in spleen (Tables 1 and 2). This confirms previous observations on absolute frequencies of LPS-reactive cells (11) by demonstrating that a considerable fraction of B cells in all lymphoid organs is the target for mitogenic stimulation. Moreover, these data suggest that LPS-dependent clonal expansion of B cells in the various lymphoid organs reveals similar potentialities to switch to the synthesis of the different heavy chain isotype.

Preliminary experiments using spleen and BM cells, detecting IgG2a- and IgG2b-secreting cells, rather than all IgG2 PFC as in the above experiments, have shown that both these (sub)classes are produced in LPS-stimulated clones.

3.2 Frequencies of background Ig-secreting cells in various lymphoid organs secreting IgM, IgG1, IgG2, IgG3 or IgA

By means of the protein A plaque assay, frequencies of background Ig-secreting cells were determined in the same organs as tested in the limiting dilution assay for LPS-inducible B cells. Thus, we tested the spleen, BM, MLN of young adult BALB/c mice and the spleen of age-matched athymic BALB/c nude mice.

Absolute frequencies of Ig-secreting cells were highest in the spleen (Table 3). Most of the splenic Ig-secreting cells produced IgM. These IgM-secreting cells were more frequent in the spleen of athymic mice than in the spleen of thymus-bearing mice. IgG1-, IgG2-, IgG3- and IgA-secreting cells were far less frequent in the spleen than IgM-secreting cells. IgA-secreting cells were more frequent in the spleen of thymus-bearing mice than in the spleen of athymic nude mice. The frequency of IgG1-, IgG2- and IgG3-secreting cells was about the same in the spleen of both groups of mice.

In the 8-week-old mice tested, the absolute frequency of total Ig-secreting cells in the BM and MLN was lower than in spleen. The heavy chain isotype distribution of the Ig-secreting cells was also found to be different from that in spleen. The most striking features were the relatively frequent occurrence of IgG2-secreting cells in BM and MLN and the low incidence of IgM-secreting cells in the latter organ.

Table 3

Frequencies of background Ig-secreting cells in spleen, BM and MLN

Mouse strain and organ[a]	IgM	IgG1	IgG2	IgG3	IgA	Total
BALB/c nude spleen	1 in 530[b]	1 in 24,000	1 in 2,500	1 in 16,000	1 in 17,000	1 in 408
BALB/c spleen	1 in 900	1 in 19,000	1 in 2,600	1 in 14,000	1 in 7,000	1 in 567
BALB/c BM	1 in 4,600	1 in 30,000	1 in 5,500	1 in 32,000	1 in 11,000	1 in 1,803
BALB/c MLN	1 in 16,000	1 in 31,000	1 in 6,300	1 in 32,000	1 in 23,000	1 in 3,047

a. Background Ig-secreting cells were determined in 8-week-old unprimed BALB/c athymic nude mice and BALB/c thymus-bearing mice.

b. Figures represent the ratio of IgM-, IgG1-, IgG2-, IgG3- or IgA-secreting cells to the total number of nucleated cells. Each figure is the arithmetic mean of the data obtained from 10 individually tested mice. The SEM of the figures varies between 8 and 23%.

4. *DISCUSSION*

The data presented in this report show that activation of single murine B cells with LPS leads to the development of clones secreting IgM, IgG1, IgG2, IgG3 and IgA. The expression of each of these heavy chain isotypes by Ig-secreting clones occurs with a relatively constant frequency, independently of the organ from which the cells were obtained, and of whether the cells were obtained from normal or athymic nude mice. The T independence of C_H gene expression in Ig-secreting cells (12) parallels the T independence of the heavy chain isotype expression at the surface of B cells (13).

The frequency of B cells that can be activated by LPS to grow in clones of Ig-secreting cells was not markedly different in the various lymphoid organs tested. The figures range from 5-20% of all B cells, being maximal in spleen and BM, and minimal among TDL (Tables 1 and 2).

This indicates that LPS-reactive B cells constitute a sizable and relatively constant proportion of the B cell population of each lymphoid organ, in spite of differences in life-span (14), surface markers (13,15) and functional properties (16) between these different B cell populations. These observations confirm previous results (11), also in respect to the lower absolute frequencies obtained in BALB/c mice, as compared to other mouse strains.

IgG-secreting cells arise in LPS-stimulated cultures by a 'switch' in C_H gene expression in cells that initially secrete IgM. The evidence for this is based upon probability analysis of the development of IgG-secreting cells in cultures of single IgM clones (3) and upon transfer and further culture of single, IgM-secreting cells (17). Furthermore, addition of anti-IgM antibodies to the LPS-activated cell prevents not only the development of IgM clones, but also of IgG clones (10,18). Finally, in LPS-activated cultures, initially only cytoplasmic IgM-positive cells appear; subsequently, cells positive for both cytoplasmic IgM and cytoplasmic IgG, while finally, cells positive for cytoplasmic IgG only predominate (19,20). This switch in C_H gene expression from IgM to other heavy chain isotypes can occur independently of T cell help and macrophages (12) and is thus independent of a local microenvironment. During the switch from IgM to IgG secretion, the surface IgM is lost, and membrane-bound IgG is expressed that serves as a target for the inhibitory action of anti-IgG antibodies (10,18). Interestingly, it appears that only one isotype (other than IgM and IgD) can be simultaneously expressed by single cells (18,21), although no firm indications exist as to the ability of normal cells to 'switch' more than once.

These observations can now be well understood in genetic terms. At the DNA level, expression of C_H genes other than μ or δ requires a second rearrangement which brings the same V gene that had previously been joined and transcribed with μ (and δ) close to the 5' end of the C_H gene and deletes all other C_H genes upstream from the recombination site (22). Indeed, appropriate stimulation of a single precursor B cell can yield a clonal offspring secreting IgM and IgG1 antibodies of the same specificity (23,24) and the same idiotype (25). Switch rearrangements are intimately connected with cell activation and clonal expansion, as shown in LPS-activated B cells. Thus, most, if not all, LPS-reactive B cells have been shown to bear IgM or IgM and IgD at the surface (26,27), and several mitotic cycles are required

before IgG production can be detected (28). The expression of IgG secretion (3) and likely, therefore, the switch rearrangement event is, however, not fixed in time, and the progeny of single precursors may contain variable numbers of switched cells after similar periods of clonal expansion (3). It also appears that not all clones switch under these experimental conditions. As shown here, even postulating that the various isotypes occur in different clones, roughly 15% of all IgM clones do not produce other Ig classes. It is unlikely that this high percentage could be accounted for by IgE-secreting cells, the only isotype we have not studied here. The total frequency of LPS-reactive B cells switching to IgG secretion reported before was only about 10% of all IgM-secreting clones (3), but this was due to the fact that only IgG1 and IgG2a PFC were detected.

It is striking that the frequency of switches from IgM to each of the other isotypes is so constant in LPS-activated B cells from different sources. In the various experiments, the IgM-secreting cells most frequently switched to the synthesis of IgG2 and IgG3, namely 30-50% of all clones (Tables 1 and 2). Switching of LPS-activated cells to IgG1 and IgA occurred only in a minority of the clones. Switching to IgG1 secretion was observed in 4-11% of the clones, while switching to IgA secreting was found in as few as 1% of the IgM-secreting clones (Tables 1 and 2). It is striking that this sequence parallels, to some extent, the order of C_H genes in the chromosome as to the direction of transcription, which has been reported to be $\gamma 3$, $\gamma 1$, $\gamma 2b$, $\gamma 2a$, ε and α (29, and Honjo, personal communication). This would suggest that the probability of switch rearrangements in LPS-reactive B cells decreases in a gradient along the chromosome. In contrast, the low frequency of IgG1 switches indicates a strong selectivity in those rearrangements. This could be determined either by precommitment of B cells in various subsets to express different C_H genes, or by regulatory signals given to the cells by the stimulus maintaining clonal expansion. As argued before (30), the latter alternative appears more likely at the present time, since LPS-reactive cells which normally produce high IgG3 and low IgG1 can be induced to high IgG1 and no IgG3 if provided with specific T helper cell signals (30).

Previous experiments have suggested a direct relationship between the relative numbers of IgM- and IgG-secreting, LPS-reactive clones and the relative numbers of IgM- and IgG-secreting background PFC in the spleen (3). The present studies, involving frequency determinations of LPS-reactive B cells and background PFC in different lymphoid organs, do not confirm this suggestion. The heavy chain isotype distribution of the Ig-secreting cells was found to vary in the different lymphoid organs (Table 3). The class distribution of Ig-secreting, LPS-induced B cell clones, on the other hand, is rather independent of the B cell source (Tables 1 and 2). This reinforces the suggestion that the expression of the different heavy chain isotypes in Ig-secreting cells is subject to extraneous regulating influences (31). Indeed, the relative numbers of IgM-, IgG- and IgA-secreting cells in lymphoid organs can be influenced by a variety of factors such as type and dose of antigen and route of administration, the use of adjuvants, and the availability of regulatory T cells (31-33).

The present experiments, as well as our previous results on the control of isotype expression by LPS and specific helper cells (30), were performed in the absence of Ig receptor recognition of antigen indicating that the control of C_H gene expression is not primarily exerted via clonally distributed receptors. The same conclusion on the importance of the functional

properties of the carrier in the class distribution of anti-hapten antibodies has recently been reached by others (34). This emphasizes that polyclonal activation of B cells provides the methodology of choice to study these questions.

We thank Margaretha Tuneskog and Rianne Preesman for excellent technical assistance, Ms. Luciana Forni for providing the rabbit anti-mouse IgM, IgG1 and IgG2 antisera, Drs. A.A. de Freitas and I. Heron for thoracic duct cannulation and Mrs. Cary Meijerink-Clerkx for typing the manuscript.

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CHAPTER VIII

FREQUENCY ANALYSIS OF FUNCTIONAL IMMUNOGLOBULIN C AND V GENE EXPRESSION IN MURINE B CELLS AT VARIOUS AGES

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In: J. Immunol. 131:1629-1634 (1983)

ABSTRACT

The frequencies of lipopolysaccharide (LPS)-reactive B cells and their antibody specificity repertoire have been determined in the spleen and bone marrow (BM) of mice at different ages. A limiting dilution culture system was employed that allows the growth and development of every LPS-reactive B cell into a clone of IgM-secreting cells, that are capable of switching to other Ig heavy chain isotypes (C gene expression). The secretion of IgM and IgG1 was assessed in the protein A plaque assay, whereas specific IgM antibody-secreting cells (V gene expression) were detected with the use of plaque assays specific for various heterologous erythrocytes and sheep red blood cells (SRBC) coupled with a number of different haptens.

The frequencies of LPS-reactive B cells in the spleen and BM of C3H/Tif, C57BL/Ka, BALB/c and CBA/Rij mice appeared to be similar in 6- to 12- and 100-wk-old animals, as was the switch frequency to IgG-secretion in three strains tested. Moreover, no age-related changes were observed in the frequencies of antigen-specific B cells within the pool of LPS-reactive B cells in the spleen and BM of C57BL/Ka mice. The frequencies ranged from 1 in 10 to 1 in 20 for NIP_4- and NNP_2-SRBC, from 1 in 50 to 1 in 100 for TNP_{30}-SRBC and from 1 in 1000 to 1 in 4000 for SRBC, HRBC and GRBC. The specificity repertoire of the 'spontaneously' occurring ('background') IgM-secreting cells in the spleen and BM on the other hand, did differ between young and old C57BL/Ka mice. During aging the frequencies of the tested specificities decreased in the spleen but increased in the BM. Our data indicate that in unintentionally immunized mice the clonal selection of B lineage cells by antigen takes place at the level of the mature, antigen-reactive B cell.

INTRODUCTION

It has been extensively demonstrated that whatever the underlying mechanisms, both humoral and cell-mediated immune functions decline in an age-related fashion (reviewed in References 1-4). Still, there is controversy concerning potential B cell functions as measured by *in vitro* stimulation with polyclonal activators such as lipopolysaccharide (LPS). In several studies, a loss of reactivity has been reported with increasing age (5-9), al-

though in other investigations no difference was found at all (10-13). It is not clear, however, whether the reported defect in B cell reactivity is due to a decreased number of reactive lymphocytes (quantitative defect) or to an altered capacity of individual cells to divide that would then lead to lower responses (qualitative defect), although some data are in favor of the former (11,14). The culture conditions employed in the different studies are important factors when deciding on the apparent discrepancy. Principally, the proliferative responses of the B lymphocytes should be directly related to the number of potentially responding cells (15); this condition was not always met in the above mentioned literature. With the introduction of the limiting dilution assay in which every mitogen- or antigen-activated B cell will grow and develop into a clone of Ig-secreting cells (16,17), and with the availability of the protein A plaque assay for detection of all cells secreting Ig of a specific isotype (18), it has become possible to assess the functions of single B cells in aging mice at the clonal level.

Andersson et al. (7) stimulated murine B cells *in vitro* under limiting dilution conditions with the mitogen LPS, which polyclonally stimulates about one-third of all mouse B cells to proliferate and to secrete the antibodies that these cells are committed to produce. Their data clearly point to an age-related decrease of the overall capacity of mouse spleen cells to produce IgM-secreting clones upon stimulation with LPS. Preliminary studies in our laboratory did not confirm this, so we decided to determine the number of LPS-reactive B cells in four mouse strains at various ages and analyze the capacity of these stimulated B cells to switch from IgM to another isotype. Furthermore, we analyzed part of the antibody specificity repertoire of these LPS-reactive B cells in plaque assays detecting antibodies of various specificities. The same was done for the 'spontaneously' occurring ('background') IgM-secreting cells to gain more insight into the establishment and maintenance of the antibody repertoire with increasing age.

The results presented in this paper show that the frequency and the antibody specificity repertoire of the LPS-reactive B cells in the spleen and bone marrow (BM) of 6- to 12-wk-old and 100- to 110-wk-old mice is quite similar and that these B cells have an equal capacity to switch from IgM to IgG1. The pattern of 'background' Ig-secreting cells in the spleen and BM is less consistent which is proved by the changing frequencies of the various antibody specificities.

MATERIALS AND METHODS

Animals

Female BALB/c, and male CBA/BrARij and C57BL/KaLwRij mice of various ages were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. Female and male C3H/Tif mice of various ages were obtained from GL. Bomholtgård, Ry, Denmark, and female Lewis rats, 4 wk of age, were obtained from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands. The mice had been barrier-maintained from birth under specific pathogen-free or clean conventional conditions and were either used within 1 day after delivery or kept in laminar flow hoods and used at the appropriate age. All mice tested were free of symptoms of lymphoreticular malignancies.

Mitogen

The mitogen LPS B from *Escherichia coli* (026:B6; Difco Laboratories, Detroit, MI) was used in the cultures at the previously determined optimal concentration of 50 µg/ml.

Cells

Thymus, spleen and BM cell suspensions were prepared as described (19). The viable cells were counted by trypan blue exclusion, and the total number of nucleated cells were counted with a Coulter counter model B (Coulter Electronics Ltd., Harpenden, Herts, UK).

Frequency determinations of LPS-reactive B cells and 'background' Ig-secreting cells

The frequencies of LPS-reactive B cells secreting IgM, IgG1 or IgM antibodies specific for 6 different antigens were estimated by the method originally described by Andersson et al. (17). Varying numbers of spleen cells (maximally 6000) and BM cells (maximally 10,000) were cultured in 96-well tissue culture plates (Costar 3596; Costar, Cambridge, MA) together with 7.2×10^5 irradiated (0.1 Gy) rat thymus cells (20) to support growth and 50 µg/ml LPS in 0.2 ml RPMI 1640 medium supplemented with glutamin (4 mM), penicillin (100 IU/ml), streptomycin (50 µg/ml), 2-mercaptoethanol (5×10^{-5} M), and fetal bovine serum (20%) (lot B 663903 02; Boehringer Mannheim GmbH, Mannheim, FRG), specifically selected for growth-supporting properties and low endogenous mitogenic activity. Routinely, 32 replicate cultures were set up for each cell concentration; control cultures did not contain mouse lymphoid cells but did contain rat thymus cells. The cultures were assayed on day 5 for total IgM plaque-forming cells (PFC) and for IgM PFC specific for several types of heterologous erythrocytes or sheep red blood cells (SRBC) coupled with different haptens. IgG1 PFC were assayed on day 7 of culture; the maximum number of IgM and IgG1 PFC observed in control cultures was different in different experiments and ranged from 10 to 25 and from 0 to 10 PFC per culture for IgM and IgG1, respectively. Control cultures, assayed with the different types of heterologous erythrocytes, never contained more than 5 antigen-specific PFC. In the limiting dilution assay, cultures were scored as positive when they yielded more than 10 PFC above the maximum number of PFC found in the control cultures. The frequencies of 'background' antigen-specific IgM-secreting cells in spleen and BM *in vivo* were determined in the appropriate plaque assays and calculated as the ratio of specific IgM antibody-secreting cells to the total number of IgM-secreting cells. BM PFC were determined in the femoral BM; following Benner et al. (19), we have adopted a conversion factor of 7.9 to calculate the number of PFC in the total BM from the number of PFC found in two femurs.

Plaque assays for Ig- and antibody-secreting cells

Ig-secreting cells were assayed by the hemolytic plaque assay as described by Gronowicz et al (18) and modified by Van Oudenaren et al. (21), with the use of *Staphylococcus aureus* protein A (Pharmacia Fine Chemicals, Uppsala, Sweden)-coated SRBC and specific rabbit anti-mouse-IgM and anti-mouse-IgG1 antibodies (kindly provided by Dr. C. Martinez, Institut Pasteur, Paris, France) and guinea pig complement (Behringwerke, Marburg Lahn, FRG). PFC directed against SRBC, horse (HRBC) and goat (GRBC) erythrocytes, 4-hydroxy-5-iodo-3-nitrophenyl (NIP)-, 4-hydroxy-3,5-dinitrophenyl (NNP)- and 2,4,6-trinitrophenyl (TNP)-SRBC were measured as described (22). NIP and NNP were coupled to SRBC by adding 0.4 or 4 mg and 0.2 or 2 mg of the hapten succinimide active esters (Biosearch, San Rafael, CA) per milliliter of washed

and packed SRBC. The coupling procedure was performed essentially as described by Pohlit et al. (23). These haptenated SRBC are referred to as $NIP_{0.4}$-SRBC, NIP_4-SRBC, $NNP_{0.2}$-SRBC and NNP_2-SRBC, respectively. TNP was coupled to SRBC by the method of Rittenberg and Pratt (24) by adding 3 or 30 mg 2,4,6-trinitrobenzene sulphonic acid (Eastman Kodak Co., Rochester, NY) per milliliter of washed and packed SRBC. They are referred to as TNP_3-SRBC and TNP_{30}-SRBC, respectively. Throughout all experiments SRBC, HRBC and GRBC from a single donor were used. All plaque assays were performed in liquid medium in Cunningham chambers (25).

RESULTS

Frequencies of total LPS-reactive B cells in spleen and BM at various ages

We employed culture conditions that allowed every growth-inducible B cell to grow and mature into a clone of Ig-secreting cells, to culture spleen and BM cells. This was done for C3H/Tif, C57BL/Ka, BALB/c and CBA/Rij mice

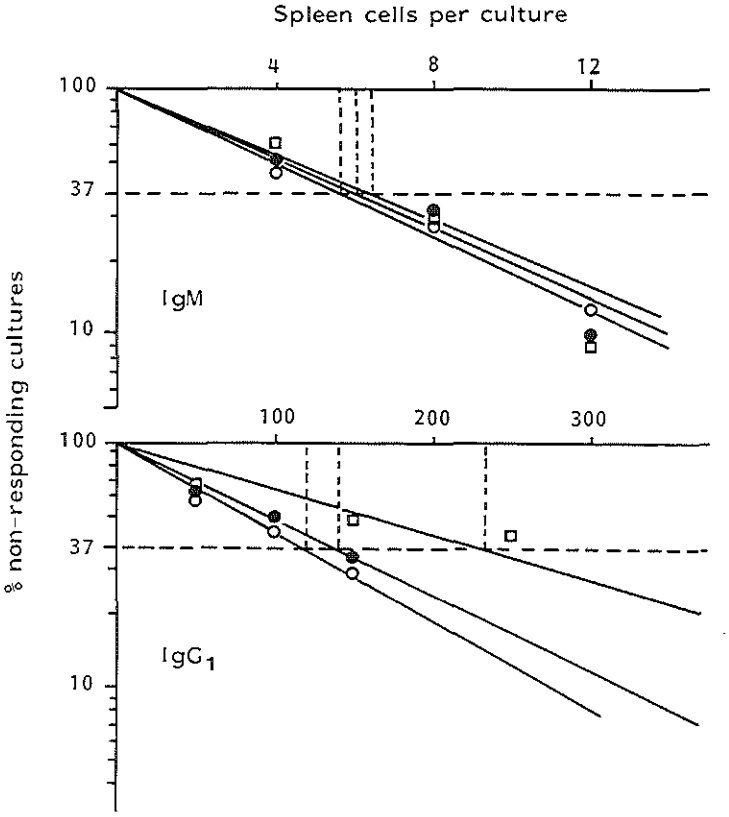

Figure 1. Limiting dilution analyses of LPS-reactive B cells in the spleens of C3H/Tif mice of 6 to 12 (●), 50 (○) and 100 (□) wk of age. Varying numbers of spleen cells were added per culture (32 cultures per experimental point). Clones of IgM- and IgG1-secreting cells were determined with the protein A plaque assay on day 5 and 7, respectively.

of various ages. LPS was used as a mitogen to determine the frequencies of LPS-reactive B cells developing into clones of IgM- and IgG1-secreting cells.

Figure 1 shows the results of such limiting dilution analyses of spleen cells of 6- to 12-, 50- and 100-wk-old C3H/Tif mice. Apparently, there is no major difference in the frequencies of IgM- and IgG1-secreting clones in these three age groups. Earlier experiments (26) indicated that IgG1-secreting cells develop as subclones within clones that initially secrete IgM. Thus, the relative 'switch frequency' can be calculated by dividing the absolute frequency of IgG1-secreting clones by the absolute frequency of IgM-secreting clones, which was 4, 5 and 3% in the three age groups, respectively. Thus, neither the number of LPS-reactive B cells in the spleen nor the relative switch frequency for IgG1 alter significantly with increasing age. In addition, the number of clones of Ig-secreting cells generated in cultures that contained one responsive B cell on average, did not appear to be different in the experimental groups and was calculated as 40 to 60 PFC after 5 days of culture. Essentially the same results were obtained for BM B cells (Table 1). Also, analyses of C57BL/Ka, BALB/c and CBA/Rij spleen and BM cells did not reveal consistent age-related differences in the frequencies of LPS-reactive B cells. There was some difference in the LPS-reactivity between the mouse strains used. C3H/Tif and C57BL/Ka mice showed the same frequency of LPS-reactive B cells, which was about 1 in 6 for spleen and about 1 in 35 for BM. BALB/c and CBA/Rij mice showed a lower response: 1 in 15 to 1 in 20 for spleen and 1 in 100 and 1 in 50 for BALB/c and CBA/Rij BM, respectively. The percentage of surface Ig-positive cells in the spleen and BM was roughly the same in the four mouse strains tested, and appeared to be quite constant over the lifespan of the mice (data not shown). The total number of viable nucleated cells in the spleen of young and old C57BL/Ka mice was $(1.02 \pm 0.06) \times 10^8$ and $(1.01 \pm 0.08) \times 10^8$, respectively, whereas in the BM these figures were $(2.0 \pm 0.04) \times 10^8$ and $(2.1 \pm 0.08) \times 10^8$, respectively.

B cells from the spleen and BM of young and old C57BL/Ka and CBA/Rij mice were found to be equally capable of switching to IgG1. In these two strains the switch percentages from IgM to IgG1 secretion for spleen cells from young and old mice were 6 and 6%, and 7 and 8%, respectively. For BM of young and old mice these figures were 7 and 8%, and 8 and 10%, respectively (Table 1).

Frequencies of LPS-reactive antigen-specific B cells in spleen and BM at various ages

To gain insight into the antibody specificity repertoire of young and old mice, we determined, under conditions of limiting dilution, the frequencies of LPS-reactive B cells specific for 6 different test antigens. This was done with the spleen and BM of 6- to 12- and 110-wk-old C57BL/Ka mice. Table II shows the results of three independent experiments in which we determined the frequencies of B cells specific for the heterologous erythrocytes of sheep, horse and goat. Essentially no age-related differences were found in the frequencies of these antigen-specific B cell precursors within the total pool of LPS-reactive B cells. The same conclusion was reached in similar experiments in which spleen and BM cells were tested for B cell clonal precursors specific for either NIP_4-, NNP_2- or TNP_{30}-haptenated SRBC (Table II).

Table 1

Frequencies of LPS-reactive B cells in spleen and BM of four different mouse strains at various ages as determined by limiting dilution analysis

Organ	Ig isotype	C3H/Tif 6-12 (wk old)	C3H/Tif 50 (wk old)	C3H/Tif 100 (wk old)	C57BL/Ka 6-12 (wk old)	C57BL/Ka 100 (wk old)	BALB/c 6-12 (wk old)	BALB/c 50 (wk old)	BALB/c 100 (wk old)	CBA/Rij 6-12 (wk old)	CBA/Rij 100 (wk old)
Spleen	IgM	1 in 6[a] (4;6;6;9)	1 in 6 (3;6;9)	1 in 9 (6;12)	1 in 6 (5;6;6;7)	1 in 7 (6;6;7;8)	1 in 18 (15;17;18;20)	1 in 13 (11;15)	1 in 8 (8)	1 in 13 (10;13;16)	1 in 15 (13;17)
	IgG1	1 in 135 (111;139;155)	1 in 116 (90;118;140)	1 in 267 (241;293)	1 in 103 (103)	1 in 125 (67;183)	nd[b]	nd	nd	1 in 185 (185)	1 in 197 (197)
BM	IgM	1 in 35 (30;34;37;40)	1 in 33 (33)	1 in 61 (54;68)	1 in 35 (30;32 37;40)	1 in 35 (25;34;36;46)	1 in 100 (90;90;120)	1 in 96 (96)	1 in 90 (90)	1 in 56 (36;76)	1 in 62 (62)
	IgG1	1 in 395 (293;497)	1 in 280 (280)	1 in 641 (636;646)	1 in 494 (478;509)	1 in 439 (333;545)	nd	nd	nd	1 in 660 (660)	1 in 591 (591)

a. Frequencies were calculated as a fraction of all viable nucleated cells. Figures represent the arithmetic mean of the data from different experiments. The figures from the individual experiments are given in parentheses. In each experiment 2 or 3 mice were used.

b. nd = not determined.

Table II

Frequencies of LPS-reactive B cells specific for SRBC, HRBC, GRBC, NIP_4-SRBC, NNP_2-SRBC and TNP_{30}-SRBC in spleen and BM of young and old C57BL/Ka mice as determined by limiting dilution analysis

Organ	Antigen	6- to 12-wk-old	110-wk-old
Spleen	SRBC	1 in 1985[a] (1506;1666;2783)	1 in 1629 (1190;1351;2347)
	HRBC	1 in 2613 (1760;2678;3402)	1 in 1919 (1351;1697;2708)
	GRBC	1 in 3997 (3333;4087;4571)	1 in 3125 (3040;3083;3253)
	NIP_4-SRBC	1 in 22 (20;22;25)	1 in 21 (15;21;28)
	NNP_2-SRBC	1 in 20 (18;19;23)	1 in 24 (20;26;26)
	TNP_{30}-SRBC	1 in 81 (75;77;90)	1 in 88 (72;91;100)
BM	SRBC	1 in 1079 (887;1150;1199)	1 in 1543 (1041;1246;2342)
	HRBC	1 in 2107 (1358;2074;2889)	1 in 2167 (1246;1641;3614)
	GRBC	1 in 4951 (2732;3159;8960)	1 in 4337 (2151;4044;6817)
	NIP_4-SRBC	1 in 9 (6;9;11)	1 in 11 (10;11;12)
	NNP_2-SRBC	1 in 11 (9;12;13)	1 in 11 (8;12;13)
	TNP_{30}-SRBC	1 in 49 (45;47;55)	1 in 73 (60;69;91)

a. The frequencies of antigen-specific B cells were calculated as a fraction of all LPS-reactive B cells, which was 1 in 6.2 $\pm$ 0.4 and 1 in 6.9 $\pm$ 0.4 viable nucleated cells in young and old spleen, respectively. In young and old BM these frequencies were 1 in 34.0 $\pm$ 2.0 and 1 in 40.5 $\pm$ 6.3 viable nucleated cells, respectively. The figures represent the arithmetic mean of the data from various experiments. The figures from the individual experiments are given in parentheses. In each experiment 2 or 3 mice were used.

Frequencies of 'background' antigen-specific IgM-secreting cells in spleen and BM at various ages

By means of the protein A plaque assay and antigen-specific plaque assays with the use of a panel of 9 different target cells, the frequencies of 'background' antigen-specific IgM-secreting cells were determined in the spleen and BM of 12-wk-old and 110-wk-old C57BL/Ka mice. The results, given in Table III,

Table III

Relative frequencies of background IgM-secreting cells specific for 9 different antigens in the spleen and BM of young and old C57BL/Ka mice

Antigen	Spleen		BM	
	12-wk-old	110-wk-old	12-wk-old	110-wk-old
SRBC	1 in 1736(1262;2386)[a]	1 in 6084(4455;8310)	less than 1 in 1953	less than 1 in 3220
HRBC	1 in 1645(1006;2691)	1 in 2008(1478;2730)	less than 1 in 1160	less than 1 in 1874
GRBC	1 in 2799(2266;3457)	1 in 6992(4546;10,754)	less than 1 in 1972	less than 1 in 4680
NIP_4-SRBC	1 in 42(36;48)	1 in 85(73;100)	1 in 616(416;912)	1 in 208(166;260)
$NIP_{0.4}$-SRBC	1 in 76(64;89)	1 in 187(144;244)	1 in 1704(1349;2152)	1 in 438(351;548)
NNP_2-SRBC	1 in 45(38;52)	1 in 94(78;114)	1 in 674(593;766)	1 in 285(226;359)
$NNP_{0.2}$-SRBC	1 in 112(93;134)	1 in 426(362;501)	less than 1 in 1930	1 in 688(516;917)
TNP_{30}-SRBC	1 in 150(116;193)	1 in 228(178;294)	1 in 1293(1093;1530)	1 in 458(349;603)
TNP_3-SRBC	1 in 1112(780;1585)	1 in 1316(963;1797)	less than 1 in 2244	1 in 2209(1739;2805)

a. The figures (geometric mean ± 1 SEM) represent the ratio of specific IgM antibody-producing cells to the total number of IgM-secreting cells as determined in the protein A plaque assay. In the spleens of 12-wk-old and 110-wk-old mice 677 (± 1 SEM 559-819) and 5985 (± 1 SEM 4960-7223) IgM-secreting cells were found per 10^6 nucleated cells, respectively. In the BM of these mice 575 (± 1 SEM 492-671) and 1895 (± 1 SEM 1629-2206) IgM-secreting cells were found per 10^6 nucleated cells, respectively (n = 13).

show that the frequencies of B cells specific for heterologous erythrocytes in the spleen demonstrated a tendency to be lower in 110-wk-old mice than in 12-wk-old mice. The same holds true for most of the frequencies of B cells reactive with the various haptenated SRBC tested. As far as the frequencies of IgM-secreting cells specific for haptenated SRBC are concerned, the pattern of the BM was different. In the BM of the 110-wk-old mice, IgM-secreting cells specific for the haptenated SRBC were more frequent. The differences found between young and old mice never exceeded a factor of 4, and mostly varied between 2 and 3. The number of IgM-secreting cells per 10^6 nucleated cells in the spleen and BM of old mice, as compared with young mice, was 9 and 3 times higher, respectively (legend to Table III).

DISCUSSION

The data presented in this report indicate that in young and old mice a similar proportion of B cells can be activated by LPS. Because our assay is highly dependent on irradiated rat thymocytes, 2-mercaptoethanol, and 20% fetal bovine serum and bypasses autologous cell-cell interactions and soluble factor induction, it cannot be excluded that other cell types than the LPS-reactive B cells may be affected in aged animals, so that antigen-induced immune responses may decrease during aging. The LPS-reactive B cells were found to be equally capable of switching from IgM-secretion to IgG1-secretion. The functional expression of the part of the V gene repertoire of newly formed LPS-reactive B cells that can be determined with our panel of heterologous erythrocytes and differently haptenated SRBC also does not change during aging. The repertoire of 'background' Ig-secreting cells, on the other hand, does change during aging.

It is striking that we did not find an age-related decrease in the frequency of LPS-reactive B cells in all four mouse strains tested (C3H/Tif, C57BL/Ka, BALB/c and CBA/Rij). These data stand in clear contrast to those previously reported by Andersson et al. (7); although they used the same *in vitro* culture system as we did, they found a more than 100-fold reduction of the number of LPS and lipoprotein-reactive B cells in the spleen of 11- to 19-mo-old C3H/Tif mice as compared with 6- to 8-wk-old mice. This might well be due to the health of the animals used. It is known that virus-infected mice can be poorly stimulated with mitogens such as LPS (27), so that a disturbance in their immune system might well be the underlying cause of the above discrepancy. On the other hand, it cannot be excluded that differences in the batches of fetal bovine serum used in both experiments might influence the different response of the old mice. Some types of fetal bovine serum appear to have an enhancing effect, e.g., on ^{3}H-thymidine uptake, particularly when suboptimal doses of mitogen are used (28). In the experiments of Andersson et al. (7) and in those presented here, however, the fetal bovine sera used had been particularly selected for growth-supporting capacity and low endogenous mitogenic activity and in both studies LPS was used at the optimal dose of 50 μg/ml. Comparative studies with the *Salmonella abortus equi* LPS used by Andersson et al. (7) and the *Escherichia coli* LPS used in our studies did not reveal a difference in the proportion of splenic and BM B cells stimulated to clonal growth (29).

Some differences in LPS-reactivity were found between the mouse strains used. C3H/Tif and C57BL/Ka showed similar frequencies of LPS-reactive B cells in the spleen and BM. This is in agreement with earlier reports for young

mice of these strains (7,22,30). CBA/Rij and BALB/c mice displayed a lower response; the low frequencies of LPS-reactive B cells in BALB/c mice have also been reported by others (7,31). Our data concerning the LPS-reactivity of the spleen and BM of CBA/Rij ($H\text{-}2^q$) mice are much lower than those reported by Andersson et al. (7) for CBA/FÜl ($H\text{-}2^k$) mice. Thus, as previously suggested by others (32), the differences in LPS-reactivity between the mouse strains used are probably genetically controlled.

Two major explanations have been proposed to explain the impairment of B cell function during aging: a quantitative explanation, in which the lack of response is ascribed to the depletion of immunocompetent cells, and a qualitative one, in which intracellular defects prevent the relevant cells from responding to appropriate antigenic, mitogenic, or intercellular signals (4). Several groups of investigators have tried to assess B cell functions without the interference of autologous T cells. This was done because different T cell subsets are thought to impair or to increase in function with increasing age and thereby interfere with B cell responses, either quantitative (33-35) or qualitative (4). In our experiments we did not find any decrease in the frequencies of LPS-reactive B cells nor of surface Ig-positive cells (data not shown). This means that these mice displayed no loss at all of LPS-induced B cell function with age or alternatively, that they were simply not old enough for us to detect such changes. The 50% survival time of the C57BL/Ka mice used in most of our experiments, however, was 110 wk, which means that mice used in this study were really of advanced age.

Another finding in our experiments was that the number of clones of Ig-secreting cells generated in cultures that contained one responsive B cell, on average, did not appear to be different in the experimental groups (data not shown). This clone population was calculated as 40 to 60 PFC after 5 days of culture, which is in agreement with earlier reports (17,30). Taken together, our data show that in senescent mice LPS-reactive B cells occur in the same frequency as in young mice, and that these B cells, after appropriate stimulation, proliferate equally as well as the B cells of young mice. In addition, the studies of Duwe et al. (11) indicate that old spleens have normal numbers of B cells and that their proliferative capacity is not impaired as measured by B cell colony formation in soft agar after LPS-stimulation. On the other hand, Abraham et al. (14), studying the proliferative capacity of LPS-stimulated mass cultures of spleen cells from C57BL/6J mice of various ages, found an age-related decrease in LPS-responsiveness. They ascribed this decline, partly on the basis of cell cycle determinations, to a reduction in the number of responding B cells.

The present study also demonstrates that the LPS-reactive B cells from the spleen and BM of young and old C3H/Tif, C57BL/Ka and CBA/Rij mice switch equally well from IgM to IgG1 synthesis. The switch frequencies from IgM to IgG1 secretion for all ages and strains tested range from 3 to 8% in the spleen and from 7 to 12% in the BM. In the BM of the C3H/Tif strain the switching percentage is particularly higher; we can not explain this satisfactorily. It has been shown that regulatory signals given to the cells by the stimulus maintaining clonal expansion can alter the switch frequencies because LPS-reactive cells that normally produce high IgG3 and low IgG1 responses can be induced to high IgG1 and very weak IgG3 secretion if cultured together with specific helper T cells (reviewed in Reference 26). In our culture system, however, fluctuating regulating signals are probably largely

overruled by the feeder activity from the large number of rat thymocytes.

The results of the determinations of the antibody specificity repertoire of LPS-reactive B cells in the spleen and BM from 6- to 12-wk-old and 110-wk-old C57BL/Ka mice point to a constant expression of this repertoire, at least as far as tested with our panel of heterologous erythrocytes and differently haptenated SRBC. This suggests a constant expression of the B cell repertoire in the newly-formed B cells during their entire lifespan. Moreover, the mechanisms involved in the selection of the available B cell repertoire are apparently not influenced by the exogenous antigenic and/or mitogenic stimulation during their lifespan. These findings are at variance with those reported by others. Nariuchi and Adler (12) found a greatly reduced LPS-induced anti-dinitrophenyl (DNP) antibody formation by spleen cells from old mice, although the proliferative response was normal. Similar results were obtained by *in vitro* stimulation of mouse spleen cells by the T-independent antigens DNP-conjugated polyacrylamide beads (36), DNP-Ficoll (37), TNP-Ficoll (38), DNP-poly(L-lysine) (39), and the response to TNP induced by LPS and TNP-SRBC (6). There is ample evidence that the mean avidity of antibodies produced upon immunization of aged mice is lower than that of antibodies produced by young mice (6,40,41), although the opposite has also been reported (42). Our frequency determinations, although limited to a fraction of the total antibody specificity repertoire, revealed the same figures throughout the lifespan of the mice and are in agreement with those reported earlier for young mice (7,22,43,44). The frequencies of LPS-reactive B cells specific for NIP_4-SRBC and NNP_2-SRBC were twice as high in the BM as in the spleen of the same animals. We cannot, however, exclude maturation- and age-related changes in avidity, as they probably remain largely undetected in the assay system used.

The frequencies of *'background'* PFC of different antigen specificities were similar to those reported for the spleen of young C3H/Tif mice (45). In aged mice, however, these frequencies were different, calculated as the ratio of specific IgM-antibody secreting cells to the total number of IgM-secreting cells, because they decreased in the spleen and increased in the BM for most of the tested specificities. Nevertheless, in old mice the absolute numbers of these antigen specificities per spleen or BM actually did increase, because the absolute number of IgM-secreting cells increased. The differences in frequencies might be explained by the finding that T-dependent antibody formation in the BM is completely dependent on B memory cells (46), which might well have another specificity repertoire than newly-formed (LPS-reactive) B cells due to antigen-induced clonal selection and clonal expansion. On the other hand, these differences can be the reflection of age-related deteriorations of the immune system that might contribute to the decrease in the heterogeneity of the antibody response observed by others (41,47). Some recent studies on the auto-anti-idiotype response during aging might be relevant in this respect. It has been suggested that the age-related decrease in the immune response is in part a consequence of the increased down-regulation by a relatively increased auto-anti-idiotype antibody response (48,49). It has also been observed that the idiotype expression changes with age; the spectrum of TNP-specific idiotypes generated in young animals in response to TNP-Ficoll is different from the spectrum of idiotypes of aged animals (48). Another report (50) also suggests that the loss of immune competence in the splenic B cell population may be due to auto-anti-idiotypic antibody regulation, possibly inducing the activation of suppressor T cells.

It is tempting to suggest that the background repertoires of germfree

and conventionally bred normal or T cell-deficient mice are also different because of differences in T cell regulation and antigenic load. Experiments in this direction are in progress. Regardless of the mechanisms that might be involved in these overall regulatory events, our data concerning *in vitro* stimulated B cells from the spleen and BM of aging mice are indicative of an unchanged potentially available B cell repertoire, whereas the data covering the 'background' PFC, the naturally expressed antibody repertoire, suggest an overall change in the regulation of this expression during aging.

ACKNOWLEDGMENT

We thank Mrs. Cary Meijerink-Clerkx for typing the manuscript.

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CHAPTER IX

FREQUENCY ANALYSIS OF THE ANTIBODY SPECIFICITY REPERTOIRE IN MITOGEN-REACTIVE B CELLS AND 'SPONTANEOUSLY' OCCURRING 'BACKGROUND' PLAQUE-FORMING CELLS IN NUDE MICE

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In: Cell. Immunol. 92:154-162 (1985)

ABSTRACT

The antibody specificity repertoire of lipopolysaccharide (LPS)-reactive B cells has been determined in the spleens and bone marrow (BM) of C57BL/Ka athymic nude mice using a limiting dilution culture system that allows the growth and development of every LPS-reactive B cell into a clone of IgM-secreting cells. In addition, the numbers of 'spontaneously' occurring ('background') IgM-, IgG- and IgA-secreting cells as well as the 'background' IgM antibody specificity repertoire has been assessed in spleens and BM. The frequencies of antigen-specific LPS-reactive B cells of C57BL/Ka nude and thymus-bearing mice showed a great similarity and ranged from 1 in 1000 to 1 in 2500 for sheep red blood cells (SRBC), horse red blood cells (HRBC), and goat red blood cells (GRBC), from 1 in 10 to 1 in 25 for 5-iodo-3-nitrophenyl-coupled SRBC, from 1 in 15 to 1 in 150 for 4-hydroxy-3,5-dinitrophenyl-coupled SRBC, and from 1 in 70 to 1 in 140 for 2,4,6-trinitrophenyl-coupled SRBC. The specificity repertoire of the 'background' IgM-secreting cells differed from that of age-matched thymus-bearing controls and was different in young and old C57BL/Ka nude mice. Within the limitations of having assessed only a minor fraction of the total B cell antibody specificity repertoire and supposing that nude mice are largely devoid of functional T cells, the data presented suggest that the generation of the specificity repertoire of newly-formed B cells is hardly or not affected by T cells. On the other hand, T cells do affect the expression of the established repertoire, represented by 'background' immunoglobulin-secreting cells.

INTRODUCTION

A possible essential role in the establishment and maintenance of the B cell repertoire has been ascribed to T cells (1-3). Classically, athymic nude mice, thought to be devoid of functional T cells, have been used to determine T cell influences on B cell functions. *In vitro* experiments using B cell mitogens (4-7) and *in vivo* experiments employing the splenic focus technique (8) suggested that nude mice possess a potentially normal B cell system (7), equivalent in diversity to that of normal mice (8). However, until now no systematic studies have been reported that analyzed the antibody specificity repertoire in young and old nude mice, neither at the level of mitogen-reactive B cells nor at the level of 'spontaneously' occurring ('background')

immunoglobulin (Ig)-secreting cells. Such analyses would permit an evaluation of the possible role that T cells might play in the generation and expression of B cell repertoires, which might be relevant for current concepts about T cell involvement in the presumptive internal regulation of the immune system (2,3,9-11).

We analyzed the antibody specificity repertoire of the B cells in the spleens and bone marrow (BM) of young and old nude mice employing an *in vitro* limiting dilution culture system that allows the growth and maturation of every lipopolysaccharide (LPS)-reactive B cell into a clone of Ig-secreting cells. Furthermore, we determined the numbers of 'background' IgM-, IgG- and IgA-secreting cells and a part of the antibody specificity repertoire of the IgM-secreting cells in the spleens and BM of these mice. For this purpose we employed the protein A plaque assay, which potentially reveals every cell secreting Ig (12,13), as well as plaque assays detecting antibodies of various specificities.

MATERIALS AND METHODS

Animals. Female and male C57BL/KaLwRij athymic nude and normal thymus-bearing mice of various ages were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. Female Lewis rats, 4 weeks of age, were purchased from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands. The mice had been barrier-maintained from birth under specific pathogen free conditions and were used within 1 day after delivery. All mice tested were free of symptoms of lymphoreticular malignancies and showed no signs of disease upon dissection.

Cells. Spleen, BM, and thymus cell suspensions were prepared as described (14). The viable cells were counted by trypan blue exclusion, and the total number of nucleated cells were counted with a Coulter Counter (Model B; Coulter Electronics Ltd., Harpenden, Herts, U.K.).

Frequency determinations of LPS-reactive B cells and 'background' Ig-secreting cells. The frequencies of LPS-reactive B cells secreting IgM or IgM antibodies specific for one out of six different antigens were estimated by the method originally described by Andersson et al. (15). In brief, varying numbers of spleen cells (maximally 4000) and BM cells (maximally 10,000) were cultured in 96-well tissue culture plates (Costar 3596; Costar, Cambridge, Mass.) together with 7.2×10^5 irradiated (0.1 Gy) rat thymus cells (16) to support growth and 50 µg/ml *Escherichia coli* LPS (026:B6, Difco Detroit, Mich.) in 0.2 ml RPMI 1640 medium supplemented with glutamine (4 mM), sodium pyruvat (0.1 M), penicillin (100 U/ml), streptomycin (50 µg/ml), 2-mercaptoethanol (5×10^{-5} M), fetal bovine serum (20%) (lot 101108 from Sera Lab. Ltd., Sussex, U.K.), specifically selected for growth-supporting properties and low endogenous mitogenic activity. Routinely, 32 or 48 replicate cultures were set up for each cell concentration; control cultures did not contain mouse cells but did contain rat thymus cells. The cultures were assayed on day 5 for total IgM plaque-forming cells (PFC) and for IgM PFC specific for sheep red blood cells (SRBC), horse red blood cells (HRBC), goat red blood cells (GRBC) or differently haptenated SRBC. In the limiting dilution assay, cultures were scored as positive when they yielded more than 10 PFC above the maximum number of PFC observed in the control cultures, which ranged from 0 to 25 for IgM-secreting cultures and from 0 to 10 for cultures

tested in antigen-specific plaque assays. The total number of 'background' Ig-secreting cells of a given class per organ was calculated 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. (14), 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 spleens and BM were determined in the appropriate plaque assays and calculated as the ratio of specific IgM antibody-secreting cells to the total number of IgM-secreting cells. Statistical evaluation was performed using the Student *t* test.

Plaque assays for Ig- and antibody-secreting cells. The target cells were prepared and the plaque assays were performed as has been extensively described (17). Briefly, 5-iodo-3-nitrophenyl (NIP), 4-hydroxy-3,5-dinitrophenyl (NNP) and 2,4,6-trinitrophenyl (TNP) were coupled to SRBC with 4, 2 and 30 mg of the hapten per ml of washed packed SRBC, respectively. The corresponding target cells are referred to as NIP_4-, NNP_2- and TNP_{30}-SRBC, respectively.

RESULTS

Frequencies of LPS-reactive antigen-specific B cells in spleens and BM. We determined under conditions of limiting dilution the frequencies of LPS-reactive B cells specific for six different test antigens. Table 1 shows the results of three independent experiments in which the spleens and the BM of 6- to 12-week-old C57BL/Ka nude mice were tested for B cell clonal precursors specific for sheep, horse and goat erythrocytes and for NIP_4-, NNP_2- and TNP_{30}-haptenated SRBC. The same was done for the spleens and the BM of 100-week-old C57BL/Ka nude mice. Essentially, no age-related differences were found in the frequencies of these antigen-specific B cells within the pool of LPS-reactive B cells, except for the NNP_2-SRBC specific B cells, which were 6 and 2.5 times less frequent in the spleens and BM of old nude mice, respectively.

Numbers of 'background' Ig-secreting cells in spleens and BM. Using the protein A plaque assay, the numbers of 'background' IgM-, IgG-, and IgA-secreting cells were determined in the spleens and BM of 6- to 12-week-old and 100-week-old C57BL/Ka nude and normal thymus-bearing mice. The results, given in Table 2, show that in the spleens and BM of young and old nude mice IgM-secreting cells were far more numerous than cells secreting IgG or IgA. Furthermore, it was found that in the spleens of old nude mice the numbers of IgM-, IgG- and IgA-secreting cells were roughly 5, 16, and 8 times increased, respectively, compared with those of young nude mice. In the BM of old nude mice 30, 16, and 28 times higher numbers of IgM-, IgG-, and IgA-secreting cells were found, respectively. In addition, the number of IgM-secreting cells in the spleens and BM of nude mice was generally higher than in the spleens and BM of age-matched thymus-bearing controls, whereas the numbers of IgG- and IgA-secreting cells were considerably lower in nude mice, especially in the BM.

Table 1

Frequencies of LPS-reactive B cells specific for SRBC, HRBC, GRBC, NIP_4-SRBC, NNP_2-SRBC and TNP_{30}-SRBC in spleens and BM of young and old C57BL/Ka nude mice as determined by limiting dilution analysis

Organ	Antigen	Frequency of antigen-specific B cells	
		6- to 12-week-old mice	100-week-old mice
Spleen	SRBC	1 in 2304 (1783,2262,2868)	1 in 2394 (2132,2523,2527)
	HRBC	1 in 1342 (1253,1333,1440)	1 in 1811 (1351;1855;2226)
	GRBC	1 in 2251 (1662,1791,3300)	n.d.
	NIP_4-SRBC	1 in 21 (18,21,23)	1 in 23 (12,20,38)
	NNP_2-SRBC	1 in 24 (15,24,32)	1 in 144[a] (102,107,223)
	TNP_{30}-SRBC	1 in 124 (101,132,138)	1 in 128 (88,111,185)
BM	SRBC	1 in 1230 (558,1000,2132)	1 in 1496 (815,1503,2169)
	HRBC	1 in 1506 (1469,1517,1532)	1 in 1565 (1430;1525;1739)
	GRBC	1 in 1554 (780,1745,2136)	n.d.
	NIP_4-SRBC	1 in 14 (7,10,15)	1 in 10 (9,10,12)
	NNP_2-SRBC	1 in 17 (13,16,23)	1 in 42 (15,45,65)
	TNP_{30}-SRBC	1 in 80 (77,78,85)	1 in 77 (71,74,85)

Note The frequencies of antigen-specific B cells were calculated as a fraction of all LPS-reactive B cells, which was 1 in 3.0 $\pm$ 0.4 and 1 in 2.6 $\pm$ 0.4 viable nucleated cells in young and old spleens, respectively. In young and old BM these frequencies were 1 in 43 $\pm$ 5 and 1 in 53 $\pm$ 19 viable nucleated cells, respectively. The figures represent the arithmetic means of the data from various experiments. The figures from the individual experiments are given in parentheses. In each experiment two or three mice were tested.
a. $P < 0.05$.

Frequencies of 'background' antigen-specific IgM-secreting cells in spleens and BM.

By means of the protein A plaque assay and antigen-specific plaque assays the frequencies of 'background' IgM-secreting cells specific for NIP_4-SRBC, NNP_2-SRBC, TNP_{30}-SRBC, and SRBC were determined in the spleens and BM of 6-

Table 2

Numbers of background IgM-, IgG- and IgA-secreting cells in the spleens and BM of C57BL/Ka nude and thymus-bearing mice

Organ	Ig-isotype	Ig-secreting cells/organ ($\times 10^{-3}$)			
		6- to 12-week-old mice		100-week-old mice	
		nu/nu (n=16)	+/+ (n=12)	nu/nu (n=8)	+/+ (n=8)
Spleen	IgM	174 (169-179)	69 (57-84)[a]	922 (839-1013)	604 (501-730)[a]
	IgG	6.1 (5.8-6.4)	51 (41-65)[a]	96 (84-110)	103 (88-119)
	IgA	8.7 (8.0-9.5)	45 (37-55)[a]	70 (64-76)	88 (79-99)[a]
BM	IgM	19 (18-20)	115 (98-134)[a]	566 (459-698)	398 (342-463)[b]
	IgG	4.7 (4.5-5.0)	154 (137-173)[a]	76 (67-87)	706 (625-790)[a]
	IgA	6.3 (5.9-6.8)	73 (62-85)[a]	176 (159-195)	843 (771-920)[a]

Note Background Ig-secreting cells were determined with the protein A plaque assay. Figures represent the geometric mean ± 1 SE of individually tested mice. For statistical evaluation the data of the nu/nu mice were compared with those of the age-matched +/+ controls.

a. $P < 0.001$.

b. $P < 0.01$

to 12-week-old and 100-week-old C57BL/Ka nude mice. It was found that the frequencies of IgM-secreting cells specific for SRBC and the various types of haptenated SRBC in the spleens and BM of old nude mice were consistently lower than in young ones (Table 3). Moreover, the IgM-secreting cells specific for the various test antigens were always less frequent in the BM than in the spleens. Finally, when comparing nude mice with age-matched normal thymus-bearing mice, it appeared that in some cases comparable frequencies were found, while in others the IgM-secreting cells specific for the antigens tested were less or more frequent. This was most prominent for TNP_{30}-SRBC specific IgM-secreting cells in spleens and BM, and NIP_4-SRBC specific IgM-secreting cells in the BM of young mice.

DISCUSSION

Congenitally athymic nude mice have been widely used in immunological research, since these animals were thought to be devoid of functional T cells and therefore helpful in the elucidation of the role that the T cell system plays in immune responses. Recently, however, it was shown that cytotoxic and helper T lymphocytes can be generated from nude mice (reviewed in references 18,19), which raises questions about the T cell deficiency of these mice. Whatever the origin of those T cells, results obtained with nude mice will have to be evaluated in the light of their presence, although the actual contribution of those precursor T cells and effector T cells to the immune function of nude mice is difficult to evaluate. At any rate, the numbers of IgG- and IgA-secreting cells were much lower in the nude mice used in our experiments, which is indicative of their T cell deficiency.

Table 3

Relative frequencies of background IgM-secreting cells specific for NIP_4-SRBC, NNP_2-SRBC, TNP_{30}-SRBC and native SRBC in the spleens and BM of young and old C57BL/Ka nude and thymus-bearing mice[a]

Organ	Antigen	6 to 12-week-old mice		100-week- old mice	
		nu/nu	+/+[b]	nu/nu	+/+[b]
Spleen	NIP_4-SRBC	1 in 22 (21-23)	1 in 42 (36-48)[c]	1 in 38 (30-46)	1 in 85 (73-100)[c]
	NNP_2-SRBC	1 in 88 (83-93)	1 in 45 (38-52)[c]	1 in 105 (89-124)	1 in 94 (78-114)
	TNP_{30}-SRBC	1 in 475 (443-509)	1 in 150 (116-193)[c]	1 in 859 (760-952)	1 in 228 (178-294)[c]
	SRBC	1 in 1917 (1667-2204)	1 in 1736 (1262-2386)	1 in 9988 (7684-12,983)	1 in 6084 (4455-8310)[d]
BM	NIP_4-SRBC	1 in 50 (44-56)	1 in 616 (416-912)[c]	1 in 206 (171-248)	1 in 208 (166-260)
	NNP_2-SRBC	1 in 232 (190-284)	1 in 674 (593-766)[c]	1 in 431 (373-499)	1 in 285 (226-359)[c]
	TNP_{30}-SRBC	<1 in 1900	1 in 1293 (1093-1530)	1 in 1848 (1482-2303)	1 in 458 (349-603)[c]
	SRBC	<1 in 1900	<1 in 1953	<1 in 56,000	<1 in 3220

a. The figures (geometric mean ± 1SE) represent the ratio of specific IgM antibody-producing cells to the total number of IgM-secreting cells as determined in the protein A plaque assay. In the spleens of 6 to 12-week-old (n=16) and 100-week-old nude mice (n=8) 1418 (± 1 SE 1377-1418) and 4513 (± 1 SE 4107-4958) IgM-secreting cells were found per 10^6 nucleated cells, respectively. In the BM of these mice 122 (± 1 SE 115-129) and 1494 (± 1 SE 1211-1842) IgM-secreting cells were found per 10^6 nucleated cells, respectively. For the normal thymus-bearing mice (n=13) these data were for the spleens 677 (± 1 SE 559-819) and 5985 (± 1 SE 4960-7223) and for the BM 575 (± 1 SE 492-671) and 1985 (± 1 SE 1629-2206), respectively. For statistical evaluation the data of the nu/nu mice were compared with those of the age-matched +/+ controls.

b. Data from Hooijkaas et al. (17).

c. $P < 0.001$.

d. $P < 0.01$.

The results obtained with the *in vitro* limiting dilution assay clearly indicate that in C57BL/Ka nude mice the proportion of B cells that can be activated by LPS is similar to that in thymus-bearing C57BL/Ka mice (17). Moreover, in athymic and euthymic mice a comparable proportion of these newly-formed LPS-reactive B cells (20,21) is specific for the antigens that we used in our test panel consisting of heterologous erythrocytes and haptenated SRBC (17). These results suggest that the repertoire of young and old athymic mice is potentially equivalent to that of their age-matched euthymic counterparts. This conclusion was also reached for young athymic and euthymic BALB/c mice by Cancro and Klinman (8), who used an adoptive transfer system followed by splenic fragment culturing to assay the diversity of the antibody response to the hemagglutinin of an influenza virus strain. Also in experiments with young and old germfree C3H/HeCr mice raised and maintained on a chemically defined ultrafiltered low molecular weight diet, it was found that the specificity repertoire of the LPS-reactive B cells did not differ from the conventionally bred and fed counterparts (22). Apparently, during their lifespan, the establishment of the potentially available B cell repertoire is neither influenced by T cells nor by exogenous antigenic and/or mitogenic stimulation, although a possible influence exerted by low numbers of (im)mature T cells can not be excluded with certainty.

It must be noted that our LPS culture system depends highly on low-dose-irradiated rat thymocytes, 2-mercaptoethanol, and 20% fetal bovine serum, which provide a maximum of factors needed for the activation and growth of every third B cell (6,15,17). This and the fact that we performed limiting dilution experiments with low numbers of cells in the cultures make it unlikely that T cells derived from the nude spleen or BM cell suspensions influenced the clonal growth of the LPS-reactive B cells *in vitro*. Earlier experiments showed that in young BALB/c nude mice and their age-matched controls an equal proportion of the B cells were activated by LPS to grow and mature into clones of IgM-secreting cells, and that these cells were equally capable of switching to the secretion of other Ig isotypes (7).

It has been suggested that the production of 'background' Ig-secreting cells may be regulated and maintained through idiotype-anti-idiotype interactions (2). This mechanism may operate at two levels: one selecting germline idiotypes at or before the pre-B cell stage (23,24; Hooijkaas et al., submitted for publication) probably involving the influence of maternal Ig transferred to the pre- and/or neonates (25,26), the other involving T cells that take part in the establishment of antibody repertoires in the periphery. It was therefore speculated that the antibody repertoires of normal and T cell deficient mice may be different (2). The analysis of the specificity repertoire of background Ig-secreting cells might validate this hypothesis. The results of such analyses, presented here (Table 3), indicate that differences between the background repertoires of age-matched athymic and euthymic mice indeed do exist.

The established influence of age and T cell dysfunction on the background B cell repertoire is restricted to the background IgM-secreting cells. So far, we have no data available about the specificity repertoire of IgG- and IgA-secreting cells, although it has been shown that IgG and IgA antibodies can express a more diverse repertoire than those of the IgM class (reviewed in 27). It is therefore tempting to suggest that large differences may exist between 'conventional', athymic nude, 'antigen-free', and aged mice since the presence of T cells, antigenic load, and age have a profound influence on

the numbers of IgG- and IgA-producing cells (28), especially in the BM (29-33). Analysis of the repertoire of these cells and the repertoire of long-lived B memory cells - not analyzed in our studies - in normal, 'antigen-free' and nude mice of various ages are of interest with regard to this particular aspect.

Although the deficiency of functional T cells in nude mice is not complete and within the limitations of having determined only a part of the antibody specificity repertoire, our results suggest that T cells do not have a great impact on the selection of the repertoire of newly-formed B cells, while they do on the background IgM-secreting cells. This is in accordance with recent reports about the T15 idiotypic dominance of BALB/c anti-phosphorylcholine (PC) antibody responses (reviewed in reference 34). Thus selection of the repertoire of newly-formed B cells may take place independent of thymus function and does not have to be mediated by any idiotypic network dependent on significant levels of T cell function. Evidence for the existence of a network of idiotype-anti-idiotype interactions in a normal immune system has recently been given (35), although it is not yet clear what the functional relevance, if any, of such a network might be (3).

In conclusion, the data presented in this paper about newly-formed B cells and 'background' Ig-secreting cells, the latter representing the end-stage of B cell development, fit in a recently formulated concept about the sites and mechanisms of B cell repertoire selection (11). One of the mechanisms is proposed to be basically independent of T cells, to be mediated by antibodies, and to operate at the level of precursor cell expansion and BM output of competent B cells. In our analyses this level might be represented by the LPS-reactive B cells and we show that their repertoire is unaffected by T cell deficiency. Another mechanism, basically T cell dependent, is supposed to operate in the periphery and to select from the available pool of specificities represented by short-lived immunocompetent B cells, those which will persist and terminally differentiate to 'natural' antibody production. This mechanism might be reflected in the 'background' Ig-secreting cells, even found in 'antigen-free' mice (29) and influenced by the absence of T cells as we have shown in this report.

ACKNOWLEDGMENTS

We thank Dr. J.J. Haaijman and Dr. J. Radl from the Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands for their generous permission to use the old C57BL/Ka nude mice and Mrs. C.J.M. Meijerink-Clerkx for typing the manuscript.

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CHAPTER X

FREQUENCY ANALYSIS OF FUNCTIONAL IMMUNOGLOBULIN C AND V GENE EXPRESSION BY MITOGEN-REACTIVE B CELLS IN GERMFREE MICE FED CHEMICALLY DEFINED ULTRA-FILTERED 'ANTIGEN-FREE' DIET

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In: J. Immunol. 134:2223-2227 (1985)

ABSTRACT

The frequencies of lipopolysaccharide (LPS)-reactive B cells and their antibody-specificity repertoire have been determined in the spleen and bone marrow (BM) of conventional (CV) and 'antigen-free' C3H/HeCr mice of various ages. The 'antigen-free' mice were germfree (GF) raised and were fed an ultra-filtered solution of chemically defined (CD) low molecular weight nutrients, and were thus devoid of exogenous antigenic stimulation. Spleen and BM cells were grown in a limiting dilution culture system that allows the growth and development of every newly formed LPS-reactive B cell into a clone of IgM-secreting cells which are capable of switching to other immunoglobulin (Ig) heavy chain isotypes (C-gene expression). The secretion of IgM and IgG1 was determined in the protein A plaque assay, whereas *specific* IgM antibody secreting cells (V-gene expression) were detected in plaque assays specific for various heterologous erythrocytes and sheep red blood cells (SRBC) coupled with a number of different haptens.

The absolute frequency of LPS-reactive B cells and their capacity to switch to IgG1-secretion was not significantly different in 8- to 12-wk-old and 52-wk-old GF-CD mice and their age-matched CV controls. Moreover, no differences were observed in the frequencies of antigen-specific B cells within the pool of LPS-reactive B cells. These frequencies ranged from 1 in 20 to 1 in 50 for NIP_4-SRBC and NNP_2-SRBC, from 1 in 100 to 1 in 150 for $NIP_{0.4}$-SRBC, from 1 in 50 to 1 in 100 for TNP_{30}-SRBC, and from 1 in 1000 to 1 in 2000 for SRBC and horse red blood cells. Within the limitations of having determined the switching capacity of IgM to IgG1 only and having assessed only a minor fraction of the total B cell antibody-specificity repertoire, the data indicate that young and old GF-CD mice, although devoid of exogenous antigenic and/or mitogenic stimulation, generate B cells with a similar switching capacity and a similar IgM antibody-specificity repertoire as CV mice.

INTRODUCTION

Whether and how exogenous stimuli play a role in the generation of immunocompetent B cells and their antibody-specificity repertoire is not known. To

increase our insight into this matter, the germfree (GF) animal seems to be the experimental model of choice. Until now, it has been reported that mice raised under GF conditions from birth show a normal incidence of surface membrane immunoglobulin (Ig)-positive small lymphocytes in the bone marrow (BM) (1). The rate at which these cells are produced is markedly reduced as compared with that of conventionally treated controls (2). Moreover, it was shown that administration of extrinsic agents, such as antigens and irritants, can stimulate the production of virgin B cells in the BM of conventionally reared mice (3). This finding suggests that the usual rate of B lymphocyte production depends on the exposure to antigens from the normal, environmental microbial flora. However, *in vivo* antigenic challenges of GF and conventional (CV) mice revealed that GF mice are capable of generating an immune response as measured either by antibody levels in the serum (4) or by the number of antibody-secreting cells in the spleen (5). *In vitro* mitogen assays also showed comparable or even greater reactivities in the spleen of GF mice (6-8). Together, these findings suggest that the production of B cells in GF mice is quantitatively as well as qualitatively sufficient to provide for an adequate immune response to the antigens used.

The crucial question remains, however, whether the GF animals used in those experiments can really be regarded as devoid of exogenous stimuli, because it has been shown that a substantial mitogenic and/or antigenic activity can be exerted by the diet (8,9). With the successful breeding of GF mice fed an ultrafiltered solution of chemically defined (CD) low molecular weight nutrients, exogenous stimuli are reduced to the presently achievable minimum (10). We used these GF-CD mice to determine the role of exogenous stimuli in the generation of the antibody-specificity repertoire.

Previous data showed that even in those 'antigen-free' mice, the generation of IgM-secreting cells was not prevented because IgM was found in the serum and cytoplasmic IgM-positive cells could be detected in the spleen (9, 11,12). In addition, the presence of 'spontaneously' occurring ('background') IgM-, IgG- and IgA-secreting cells in the spleen and BM of GF-CD mice was demonstrated recently (13). Preliminary *in vitro* experiments revealed that these mice possess a substantial number of lipopolysaccharide (LPS)-reactive B cells. This allowed us to determine the frequency of newly-formed, LPS-reactive B cells at the clonal level, employing a limiting dilution culture system that induces every third B cell to proliferate and mature into a clone of IgM-secreting cells, which are capable of switching to the secretion of other Ig heavy chain isotypes (C-gene expression), which was assessed in the protein A plaque assay. Specific IgM-antibody secreting cells (V-gene expression) were detected in plaque assays specific for sheep red blood cells (SRBC), horse red blood cells (HRBC), and differently haptenated SRBC.

Our data indicate that, in spite of exhaustive deprivation of exogenous antigenic and/or mitogenic stimulation, young and old GF-CD mice generate comparable numbers of B cells with a similar switching capacity and a similar IgM antibody-specificity repertoire to CV mice.

MATERIALS AND METHODS

Animals. Female Lewis rats, 4 wk of age, were purchased from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands. Female C57BL/6J mice, 4- to 6-wk-old, were obtained from OLAC 1976, Blackthorn,

England. Male and female C3H/HeCr mice were reared and maintained in the Lobund Laboratory, University of Notre Dame, Notre Dame, IN under CV or GF conditions (10). The CV mice were fed natural ingredient (NI) L-485 (14), and the GF mice were fed ultrafiltered 'antigen-free' diet L-489 E14Se and LADEK 69E6. The water soluble, CD diet L-489 E14Se had a composition like that of L-489 E11 (10) except that all of the amino acids, B vitamins, and minerals were increased by 23% of their L-489 E11 concentrations at the expense of dextrose (reduced 8%). In addition, vitamin B-12 was specifically increased from its L-489 E11 level of 0.12 mg to 1.44 mg per 100 g water soluble solids. Na_2SeO_3 was specifically raised from 0.018 mg 0.096 mg per 100 g. The water solution was ultrafiltered to remove impurities above 10,000 daltons and was fed to the animals *ad libitum*. LADEK 69E6 (10), a separately filtered mixture of purified triglycerides and fat soluble vitamins, was supplied daily in a fixed quantity into dishes. The ashless filter paper bedding was consumed by the mice and proved important for normal intestinal function (10,15), so it must be considered as part of the diet.

In the experiments, 8- to 12-wk-old and 52-wk-old mice were used, respectively, and the data obtained from male and female mice were pooled. All mice tested were healthy and showed no abnormalities on dissection. It should be noted, however, that all CV and GF mice carry a leukemogenic virus, which in the C3H/HeCr strain always remains latent, and that at present no strictly GF mice are known to exist (10,16).

Cells. Spleen and BM cell suspensions were prepared as described (17). The total number of nucleated cells were counted with a Coulter counter model BZI (Coulter Electronics, Harpenden, Berks, England). Viability was tested by trypan blue dye exclusion. Cell suspensions were frozen in RPMI 1640 medium supplemented with 5×10^{-5}M 2-mercaptoethanol, 4 mM glutamin, penicillin (100 IU/ml), streptomycin (50 µg/ml), 20% fetal bovine serum (lot B 663903 02; Boehringer Mannheim GmbH, Mannheim, FRG), specifically selected for growth-supporting properties and low endogenous mitogenic activity and 10% DMSO (dimethylsulfoxide; Art. 2950, E. Merck, Darmstadt, FRG) in 2 ml glass tubes at a cooling rate of 1°C/min. Frozen cells from C3H/HeCr mice were transported from the Lobund Laboratory in Notre Dame to Rotterdam on solid carbon dioxide and were stored at -70°C. For cell culture experiments, performed within 6 months, the cells were thawed quickly. This was accomplished by gentle agitation of the tubes in a 37°C water bath until only a small lump of ice remained in each tube. These were kept on ice, and the completely thawed cell suspension was then transferred quickly to sterile 15 ml plastic tubes (Falcon 2057 tube; Becton Dickinson and Co., Oxnard, CA). Immediately thereafter, cold RPMI medium, supplemented as described above (but without DMSO), was added dropwise with a 1 min doubling time of the suspension volume. Cells were then spun down and were washed twice in fresh medium. Their viability was determined as described above.

Immunofluorescence. After freezing and thawing spleen and BM cell suspensions were examined for the presence of B cells by staining with fluorescein isothiocyanate-conjugated goat anti-mouse-IgM(Fc) serum (batch no. 35-178, Nordic, Tilburg, The Netherlands). To this end, 1×10^6 cells in 100 µl of 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (pH 7.8) were incubated with 15 µl of a 1/10 dilution of the antiserum in PBS for 30 min on melting ice. Afterwards, the cells were washed twice with 1% BSA in PBS and were finally mounted in glycerol/PBS (9:1) with 1 mg/ml phenylenediamine (pH 8) to prevent fading of the fluorescence (18).

Frequency determinations of LPS-reactive B cells. The frequencies of LPS-reactive B cells secreting IgM, IgG1, or IgM-antibodies specific for six different antigens were estimated by the method originally described by Andersson et al. (19). Varying numbers of viable nucleated spleen cells (maximally 6000) and BM cells (maximally 10,000) were cultured in 96 well tissue culture plates (Costar 3596; Costar, Cambridge, MA) together with 7.2 x 10^5 irradiated (0.1 Gy) rat thymus cells (20) to support growth, and an optimal dose of 50 µg/ml LPS B from *Escherichia coli* (026;B6; Difco Laboratories, Detroit, MI) in 0.2 ml RPMI 1640 medium supplemented as described above (but without DMSO). Routinely, 32 replicate cultures were set up for each cell concentration; control cultures did not contain mouse lymphoid cells but did contain rat thymocytes. The cultures were assayed on day 5 for total IgM-plaque-forming cells (PFC) and for IgM-PFC specific for SRBC, HRBC, or differently haptenated SRBC. IgG1-PFC were assayed on day 7 of culture. The maximal number of IgM- and IgG1-PFC observed in control cultures containing rat thymus cells but no spleen or BM cells was different in different experiments, and ranged from 10 to 25 and from 0 to 10 PFC per culture for IgM and IgG1, respectively. Control cultures assayed with the heterologous erythrocytes and haptenated SRBC never contained more than five antigen-specific PFC. In the limiting dilution assay, cultures were scored as positive when they yielded more than 10 PFC above the maximal number of PFC found in the control cultures. The frequency of reactive cells was determined by confirming the fraction of negative cultures to the zero-term of the Poisson distribution. Statistical evaluation was performed by using Student's *t* test.

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 extensively (21). For the hapten-specific plaque assays, 5-iodo-3-nitrophenyl (NIP), 4-hydroxy-3,5-dinitrophenyl (NNP), and 2,4,6-trinitrophenyl (TNP) were coupled to SRBC by adding 4, 0.4, 2, and 30 mg of the hapten per ml of washed packed SRBC, respectively. They are referred to as NIP_4-, $NIP_{0.4}$-, NNP_2-, and TNP_{30}-SRBC, respectively. Throughout all experiments, SRBC and HRBC from a single donor were used.

RESULTS

Pilot studies on freezing and thawing of spleen and BM cells

Preliminary experiments on frozen and thawed C57BL/6J spleen and BM cells revealed that under our freezing and thawing conditions, about 15 to 30% of the nucleated spleen and BM cells were recovered as viable cells, among which a normal proportion of B cells was detected by immunofluorescence staining (spleen: 40 to 50%; BM: 6 to 10%). After *in vitro* culture of 1200 viable spleen cells/culture or 6000 viable BM cells/culture in the presence of LPS, similar numbers of IgM-PFC were found on day 4 after culture as after culture of the same number of viable unfrozen cells of the same sample, namely, around 10,000 IgM-PFC/culture for spleen and 2500 PFC/culture for BM. Also, limiting dilution experiments with frozen-thawed spleen and BM cells (the assay system used for the present studies) revealed the same absolute frequency of LPS-reactive B cells as in unfrozen cells of the same sample. In both cases, a frequency of one LPS-reactive B cell among six viable nucleated spleen cells was found. For the BM, this figure was 1 in 41. Therefore, it seems justifiable to conclude that the viable cell fraction of frozen-thawed spleen and BM cells are a representative fraction of the total spleen and BM nucleated cells.

Table 1

Frequencies of LPS-reactive B cells in CV-NI C3H/HeCr mice and GF-CD C3H/HeCr mice

Organ	Ig isotype	8- to 12-wk-old		52-wk-old	
		CV-NI	GF-CD	CV-NI	GF-CD
Spleen	IgM	1 in 14[a] (4;11;17;25)	1 in 6 (4;4;5;11)	1 in 15 (3;7;20;29)	1 in 7 (3;6;8;9)
	IgG1	1 in 118 (47;188)	1 in 61 (41;81)	1 in 252 (121;383)	1 in 43 (38;48)
	switch IgM-IgG1[b]	12%	10%	6%	16%
BM	IgM	1 in 185 (93;142;240;265)	1 in 129 (52;60;110;294)	1 in 112 (105;119)	1 in 84 (56;60;74;146)
	IgG1	1 in 2814 (1423;2108;4910)	1 in 880 (795;965)	1 in 2370 (2370)	1 in 965 (785;1144)
	switch IgM-IgG1	7%	16%	5%	9%

a. Frequencies were calculated as fraction of all viable nucleated cells. Figures represent the arithmetic mean of the data from different experiments. Clones of IgM- and IgG1-secreting cells were determined with the protein A plaque assay on day 5 and day 7, respectively. The figures from the individual experiments are given in parentheses. In each experiment two or three mice were used. For statistical evaluation, the data of the GF-CD mice were compared with those of the age-matched CV-NI controls. No significant differences were found.

b. The relative switch frequency was calculated by dividing the absolute frequency of IgG1-secreting clones by the absolute frequency of IgM-secreting clones.

Table 2

Frequencies of LPS-reactive B cells specific for NIP_4-SRBC, $NIP_{0.4}$-SRBC, NNP_2-SRBC, TNP_{30}-SRBC, SRBC, and HRBC in CV-NI C3H/HeCr mice and GF-CD C3H/HeCr mice

		8- to 12-wk-old		52-wk-old	
Organ	Antigen	CV-NI	GF-CD	CV-NI	GF-CD
Spleen	NIP_4-SRBC	1 in 16[a] (6;12;20;24)	1 in 37[b] (18;55)	1 in 22 (19;23;23)	1 in 45 (25;36;74)
	$NIP_{0.4}$-SRBC	1 in 120 (94;122;143)	1 in 142 (99;152;174)	nd[c]	nd
	NNP_2-SRBC	1 in 19 (10;12;18;35)	1 in 19 (17;20)	1 in 37 (16;38;57)	1 in 27 (16;17;36;39)
	TNP_{30}-SRBC	1 in 54 (49;61;53)	1 in 27[d] (16;17;36;39)	1 in 90 (50;76;144)	1 in 84 (57;74;96;109)
	SRBC	1 in 1470 (864;977;2567)	1 in 1640 (1122;1540;2258)	nd	nd
	HRBC	1 in 1828 (1037;1962;2484)	1 in 1656 (1264;1624;2081)	nd	nd
BM	NIP_4-SRBC	1 in 9 (4;14)	1 in 10 (6;13)	1 in 7 (7)	1 in 12 (7;7;15;17)
	NNP_2-SRBC	1 in 17 (10;23)	1 in 20 (10;29)	1 in 9 (9)	1 in 26 (20;20;24;40)
	TNP_{30}-SRBC	1 in 75 (46;103)	1 in 59 (36;63;77)	1 in 51 (51)	1 in 69 (42;48;87;100)

a. Frequencies of antigen-specific B cells were calculated as fraction of all LPS-reactive B cells. The frequencies of LPS-reactive B cells were as stated in Table 1. Figures represent the arithmetic mean of the data from individual experiments. The data from the individual experiments are given in parentheses. In each experiment, two or three mice were tested.

b. For statistical evaluation, the data of the GF-CD mice were compared with those of the age-matched CV-NI controls.

c. nd = not determined.

d. $p < 0.05$.

Frequencies of LPS-reactive B cells in spleen and BM

We employed a limiting dilution culture system that allows every growth-inducible B cell to grow and mature into a clone of Ig-secreting cells to determine the frequency of LPS-reactive B cells in the frozen-thawed spleen and BM cells of C3H/HeCr mice of various ages. One group was reared and maintained under CV conditions, the other was GF and was fed a soluble, antigen-free diet. Table 1 shows the results of such limiting dilution analyses. It was calculated that the frequencies of LPS-reactive B cells in the spleen of 8- to 12-wk-old and 52-wk-old GF-CD mice were not significantly different from those found in age-matched CV mice. The same was found in the BM. No differences were observed between the number of LPS-reactive B cells in the spleen of young and old GF-CD, and of young and old CV-NI mice. The same holds true for the BM.

B cells from the spleen and BM of young and old GF-CD and CV-NI mice were capable of giving rise to IgG1-secreting clones. Earlier experiments indicated that IgG1-secreting cells develop as subclones within clones that initially secrete IgM (22). Therefore, the relative 'switch frequency' can be calculated by dividing the absolute frequency of IgG1-secreting clones by the absolute frequency of IgM-secreting clones. The IgG1 switch frequencies were 10 and 12% for the spleen of young GF-CD and CV-NI mice, and 16 and 6% for the spleen of old GF-CD and CV-NI mice, respectively. For the BM these data were 16 and 7%, and 9 and 5%, respectively (Table 1).

The percentage of surface Ig-positive cells in the frozen-thawed spleen cell suspensions of GF-CD and CV-NI mice was 48% $\pm$ 1.8 and 46% $\pm$ 2.3 for young mice, and 46% $\pm$ 3.2 and 45% $\pm$ 3.7 for old GF-CD and CV-NI mice, respectively. For the BM these data were 6% $\pm$ 0.6 and 6% $\pm$ 0.9 and 9% $\pm$ 0.6 and 8% $\pm$ 0.6, respectively.

Frequencies of LPS-reactive, antigen-specific B cells in spleen and BM

The frequencies of LPS-reactive B cells specific for six different antigens were determined under limiting dilution culture conditions. This was done for spleen and BM of young and old GF-CD and CV-NI mice. Essentially, similar numbers of antigen-specific LPS-reactive B cells were detected in the spleens of GF-CD and CV-NI mice (Table 2). The same was found for the BM. It should be noted, however, that in young and old GF-CD and CV-NI mice, the frequencies of B cells specific for NIP_4-SRBC are approximately three times higher in the BM than in the spleen.

DISCUSSION

When determining the capacity of germfree mice fed an 'antigen-free' diet to generate functional B cells, it is necessary that the height of the responses be directly related to the number of potentially responding cells. Therefore, a limiting dilution assay that allows every mitogen-activated B cell to grow and mature into a clone of Ig-secreting cells fulfills this condition. In this study we employed the limiting dilution culture system originally developed by Andersson et al. (19) which activates only newly-formed, short-lived B cells (23,24). It was found that B cells from GF-CD mice are equally capable of switching to IgG1 and of giving rise to similar proportions of antigen-specific IgM-secreting clones as B cells from CV mice.

It should be noted that our assay is highly dependent on rat thymus cells, 2-mercaptoethanol, and 20% fetal bovine serum, and bypasses autologous cell-cell interactions and soluble factor induction, which are thought to be essential for B cell activation (25). An alternative system using defined thymoma (EL4) filler cells, LPS, anti-Ig, and EL4 supernatant triggered one in eight C57BL/6 surface Ig-positive cells to produce a clone of Ig-secreting cells (25) which is less than the one in three that can be activated in our system (19-21). Because we wanted to assay as many B cells as possible for their Ig (sub)class switching capacity and specificity repertoire, and to compare the results with earlier determinations, we used this system throughout all experiments. Therefore, possible T cell dysfunctions in GF mice which might occur, according to other investigators (26,27), are bypassed in our assay system. Thus, it cannot be excluded that in old GF-CD mice, although they exhibit comparable B cell reactivities to young ones, which is in accordance with earlier aging experiments on conventional mice (21), cell types other than LPS-reactive B cells may be affected. The latter would explain why *in vivo* antigen-induced immune responses may decrease during aging (28). Our results also indicate that the decreased switching frequency from IgM to other Ig (sub)classes, which is occasionally observed in GF mice, is not due to an intrinsic B cell defect, but might be caused by altered T cell function (27).

Cryopreservation of lymphocytes is increasingly performed and accumulating data indicate that a normal blastogenic or PFC response to mitogens is retained after freezing and thawing of murine and human lymphoid cell suspensions (29,30). Our protocol of freezing and thawing revealed comparable cell losses in BM and spleen suspensions of both CV-NI and GF-CD mice and showed the same proportion of sIg^+ cells before and after freezing. The frequency of LPS-reactive B cells showed some variation, whereas in the different experiments in the BM, the frequency of LPS-reactive B cells was a little lower than the 1 in 30 to 40 that has been found before in C3H/Tif and C57BL/Ka mice (21). Because this occurred in both GF and CV groups, this might reflect a characteristic of the C3H/HeCr strain or a further loss of thawed LPS-reactive BM B cells during the dilution manipulations, leading to an overestimation of the number of cultured cells resulting in lower and more variable B cell frequencies. However, there is no reason to assume that selective cell losses have occurred during the total series of cell manipulations.

The present results, as are all analyses of diversity, are necessarily restricted to a fraction of the specificity repertoire. The 'indicator' antigens here are clearly advantageous because of the relatively large proportion of all antibody-secreting clones they reveal (1 to 10%), which allows their detection in the plaque assay system employed. On the other hand, it might be argued that these high frequencies reflect low affinity antibodies and provide no indications for their fine specificity, therefore being of poor value in the analyses of repertoires. However, lower precursor frequencies for haptenated heterologous erythrocytes can not be detected, because of disturbing clones reactive against the heterologous erythrocytes themselves (1 in 1000 to 2000 of the LPS-reactive B cells are reactive with SRBC or HRBC). Therefore, other assay systems analyzing culture supernatants should be employed, such as ELISA or RIA, using specific antibodies against certain idiotypes for example. It should be noted, however, that such systems also have their limitations, because anti-idiotypic antibodies have not been raised for C3H/HeCr mice so far, and because for supernatant analyses, the upper limit to the number of cells that can be cultured under limiting dilution conditions is 40 to 50 x 10^3 cells per culture of 0.2 ml (31) or 1 ml (32) at most.

For PFC analyses of Ig-secreting cells, maximally 3000 to 6000 spleen cells can be cultured per 0.2 ml because higher doses disturb the linear relationship between cell input and the log fraction of negative cultures, due to growth inhibition by crowding of cells (unpublished observations).

A striking observation in this report is the apparent similarity of the antibody specificity repertoire as expressed by B cells from young and old GF-CD mice, in spite of the reduced lymphocyte production rate in the BM of GF mice (2). This would imply that exogenous stimuli influence the B cell production rate, but do not affect the B cell antibody repertoire, which confirms and extends some earlier reports by other investigators showing similar B cell precursor frequencies for 2,4-dinitrophenyl, TNP, fluorescein, and phosphorylcholine in young adult GF and CV BALB/c mice (33,34). Also B cell immune responses upon *in vivo* immunization of GF mice (5,35,36) and other species (reviewed in references 8 and 37) that were GF raised and were maintained on normal diets were unimpaired. Thus, our data indicate that the developed CD diet (10), proven to be adequate for reproduction in successive generations of mice (15,38), does not influence the immunologic competence as determined at the level of LPS-reactive B cells, and can therefore be used for other immunologic studies, especially those in which disturbing influences of unknown antigens and/or mitogens from bacteria or food should be avoided. At the level of the 'spontaneously' occurring ('background') Ig-secreting cells, few differences were observed also when comparing the specificity repertoire of the background PFC of GF-CD and CV-NI mice. These data are reported and discussed elsewhere (13).

ACKNOWLEDMENT

We gratefully acknowledge Mrs. C.J.M. Meijerink-Clerkx for typing the manuscript, and all members of the Lobund Laboratory who in some way contributed to this work.

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CHAPTER XI

ISOTYPES AND SPECIFICITIES OF IMMUNOGLOBULINS PRODUCED BY GERMFREE MICE FED CHEMICALLY DEFINED ULTRAFILTERED 'ANTIGEN-FREE' DIET

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In: Eur. J. Immunol. 14:1127-1130 (1984)

SUMMARY

The regulation of the 'spontaneously' occurring ('background') Ig-synthesis of mice has been studied by determining the numbers of IgM-, IgG- and IgA-secreting cells and a part of the IgM antibody-specificity repertoire in spleen, bone marrow (BM) and mesenteric lymph nodes (MLN) of conventional and 'antigen-free' mice. These antigen-free mice were germfree (GF) raised and fed an ultrafiltered solution of chemically defined (CD) low molecular weight nutrients, and thus devoid of exogenous antigenic stimulation. The secretion of IgM, IgG and IgA by spleen, BM and MLN cells was assessed in the protein A plaque assay, while specific IgM antibody-secreting cells were detected by plaque assays specific for differently haptenated sheep red blood cells.

In general, antigen-free and conventional (CV) mice were found to have roughly equal numbers of IgM-secreting cells in spleen and BM. The number of IgG-secreting cells in the spleen of antigen-free mice was the same as in the spleen of CV mice, but in the BM their number was 3-5-fold decreased. About one half of the antigen-free mice did not have MLN, and in the half which did, 5 times less IgM- and more than 100-fold less IgG-secreting cells were found as compared with CV mice. The number of IgA-secreting cells in antigen-free mice was drastically decreased in all three organs tested.

The antibody-specificity repertoire of the 'background' IgM-secreting cells in the spleen and BM of the antigen-free and CV mice was much alike. This indicates that in antigen-free mice the available antibody repertoires are established independently of exogenous antigenic and/or mitogenic stimulation.

1. INTRODUCTION

The possible essential and necessary influence of exogenous antigens and/or mitogens on the development of the B cell repertoire, as expressed by 'spontaneously' occurring ('background') immunoglobulin-(Ig) or antibody-secreting cells (1), has neither been properly established nor excluded. With the successful breeding of germfree (GF) mice fed an ultrafiltered solution of chemically defined (CD) low molecular weight nutrients ('antigen-free'

mice) these exogenous stimuli are reduced to the presently achievable minimum (2,3). Previous studies using these GF-CD mice revealed both the occurrence of IgM in the serum (4) and cytoplasmic IgM antibody containing cells (cIgM cells) in the spleen (5). IgG was absent (4) or hardly detectable (6) in the serum and the number of cIgG cells in the spleen was 10-fold reduced (5). Moreover, IgA was not detected in the serum (6) and cIgA cells could not be detected in spleen, bone marrow (BM) and mesenteric lymph nodes (MLN) (5). Thus, it was concluded that complete deprivation of GF mice from exogenous antigens does not prevent the production and secretion of IgM.

In view of the potential implications of the data on the immunological activity of antigen-free mice for the contemporary thinking about internal regulation of the immune system, we decided to study the 'background' IgM, IgG and IgA secretion in various lymphoid organs of antigen-free mice. For this purpose, the protein A plaque assay was employed, as this assay potentially detects every cell secreting Ig. Furthermore, a part of the antibody specificity repertoire of the IgM-secreting cells in spleen and BM was analyzed by using plaque assays detecting antibodies of various specificities, to establish whether or not exogenous stimuli are needed to generate the repertoire of background IgM-secreting cells.

2. *MATERIALS AND METHODS*

2.1 Animals. Male and female C3H/HeCr mice were reared and maintained in the Lobund Laboratory, University of Notre Dame, Notre Dame, IN (USA) under conventional (CV) or GF conditions (2). The CV mice were fed natural ingredient (NI) L-485 (7) and the GF mice CD ultrafiltered 'antigen-free' diet L-489 E14Se and LADEK 69E6. The water-soluble, chemically defined diet L-489 E14Se had a composition like that of L489 E11 (3) except that all the amino acids, B vitamins and minerals were increased by 23% of their L489 E11 concentrations at the expense of dextrose (reduced 8%). In addition, vitamin B12 was specifically increased from its L489 E11 level of 0.12 mg to 1.44 mg per 100 g water-soluble solids. Na_2SeO_3 was specifically raised from 0.018 mg to 0.096 mg per 100 g. The water solution was ultrafiltered to remove impurities above 10 kDa and fed *ad libitum*. LADEK 69E6 (3), a separately filtered mixture of purified triglycerides and fat-soluble vitamins was measured daily into dishes. The ashless filter paper bedding was consumed by the mice and proved important for normal intestinal function (2), so it must be considered part of the diet. In the experiments 8-12-week-old and 52-week-old mice were used, respectively, and the data obtained from male and female mice were pooled. All mice tested were in a healthy condition and showed no abnormalities on dissection. It must be noted, however, that all CV and GF mice carry a leukemogenic virus, which in the C3H/HeCr strain always remains latent and that at present no strictly GF mice are known to exist (3,8,9).

2.2 Cells. Spleen, BM and MLN cell suspensions were prepared as described (10). The total number of nucleated cells were counted with a Coulter counter (Coulter Electronics, Harpenden, GB).

2.3 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 in detail (11). Briefly, 4-hydroxy-5-iodo-3-nitrophenyl (NIP), 4-hydroxy-3,5-dinitrophenyl acetyl (NNP) and 2,4,6-trinitrophenyl (TNP) were coupled to sheep red blood

cells (SRBC) with 4 or 0.4, 2 or 0.2, and 30 mg of the hapten/ml of washed and packed SRBC, respectively. These haptenated SRBC are referred to as NIP_4-SRBC, $NIP_{0.4}$-SRBC, NNP_2-SRBC, $NNP_{0.2}$-SRBC and TNP_{30}-SRBC. Throughout all experiments SRBC from a single donor were used.

2.4 Calculation of the total number of background 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. (10) 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 spleen and BM were determined in the appropriate plaque assays and calculated as the ratio of specific IgM antibody-secreting cells to the total number of IgM-secreting cells. Statistical evaluation was performed using the Student's *t* test.

3. RESULTS

3.1 Background Ig-secreting cells in spleen, BM and MLN. By means of the protein A plaque assay the total number of 'background' IgM-, IgG- and IgA-secreting cells were determined in the spleen, BM and MLN of 8-12-week-old and 52-week-old conventional C3H/HeCr mice fed natural ingredient (CV-NI), as well as in antigen-free C3H/HeCr mice (GF-CD). The results are given in Table 1. In the spleen of young GF-CD mice the total number of IgM-secreting cells was approximately half of that seen in the CV-NI controls, whereas it was the same in 52-week-old CV-NI and GF-CD mice. The similarity at 52 weeks is caused by the fact that in CV-NI mice IgM-secreting cells in the spleen decline with age, while in GF-CD mice these cells are more numerous at 52 than at 8-12 weeks. The number of IgG-secreting cells did not differ significantly between the CV-NI and GF-CD mice of both age groups. In both groups, values at 52 weeks were higher than at 8-12 weeks. With regard to the IgA-secreting cells a 4-5-fold decrease was seen both in the young and in the old GF-CD group as compared with their CV-NI controls.

In the BM of the young GF-CD mice the numbers of IgM-, IgG- and IgA-secreting cells were roughly 2-, 5- and 17-fold reduced, respectively, as compared with CV-NI controls. In the BM of the old GF-CD mice the number of IgM-PFC was approximately the same as in the age-matched CV-NI mice. However, the numbers of IgG and IgA PFC were 3 and 74 times lower, respectively. In both the CV-NI and GF-CD group the total number of IgM- and IgG-secreting cells was higher in old animals than in young ones. The number of IgA-secreting cells also increased with age in CV-NI mice, but not in the GF-CD mice. The number of IgA-secreting cells in GF-CD mice, however, was in the old group as low as in the young one.

A striking difference between the conventional and antigen-free mice was observed in the MLN. In the 8-12-week-old GF-CD group, MLN were found in only 10/20 mice and in the 52-week-old GF-CD group in only 1/10 mice. From the mice that did possess MLN the data are given. It was found that in the young GF-CD group the numbers of IgM-, IgG- and IgA-secreting cells were 5, 138 and 111 times lower than in the CV-NI mice, respectively. For the only old animal

Table 1

Numbers of background Ig-secreting cells in different lymphoid organs of CV-NI C3H/HeCr mice and GF-CD C3H/HeCr mice

Organ	Ig isotype	Ig-secreting cells/organ[a] x 10^{-3}			
		8-12-week-old mice		52-week-old mice	
		CV-NI	GF-CD	CV-NI	GF-CD
Spleen	IgM	840(804-876)[b]	365(333-402)[c,d]	553(470-651)	613(556-676)[e]
	IgG	67(59-77)	72(68-77)[e]	121(84-174)	133(120-147)[e]
	IgA	54(50-58)	13(12-14)[d]	48(33-68)	8.8(7.7-10.1)[d]
BM	IgM	106(100-114)	55(50-60)[d]	339(279-412)	277(236-327)[e]
	IgG	80(77-84)	17(16-18)[d]	171(141-208)	57(45-72)[f]
	IgA	47(40-56)	2.8(2.5-3.1)[d]	207(152-285)	2.8(2.2-3.7)[d]
MLN	IgM	2.0(1.7-2.5)	0.37(0.24-0.59)[d,g]	5.4(3.9-7.4)	1.3[f,h]
	IgG	11(8.8-14)	0.08(0.05-0.13)[d]	7.5(6.1-9.2)	0.3[d]
	IgA	10(9.0-11)	0.09(0.04-0.22)[d]	15(13-18)	0.5[d]

a. Background Ig-secreting cells were determined with the protein A plaque assay.
b. Figures represent the geometric mean $\pm$ 1 SE of 20 8-12-week-old and 10 52-week-old individually tested mice.
c. For statistical evaluation the data of the GF-CD mice were compared with those of the age-matched CV-NI controls.
d. $P < 0.001$.
e. Not significantly different.
f. $P < 0.01$.
g. In the 8-12-week-old GF-CD group MLN were found in only 10 out of 20 mice.
h. In the 52-week-old GF-CD group MLN were found in only 1 out of 10 mice.

Table 2

Relative frequencies of background IgM-secreting cells specific for NIP_4-SRBC, $NIP_{0.4}$-SRBC, NNP_2-SRBC, $NNP_{0.2}$-SRBC, and TNP_{30}-SRBC in spleen and BM of CV-NI C3H/HeCr mice and GF-CD C3H/HeCr mice

Organ	Antigen	8-12-week-old mice		52-week-old mice	
		CV-NI	GF-CD	CV-NI	GF-CD
Spleen	NIP_4-SRBC	1 in 31 (30-33)[a]	1 in 32 (30-33)[b,c]	nd[d]	nd
	$NIP_{0.4}$-SRBC	1 in 144(136-153)	1 in 127(113-143)[c]	1 in 107 (94-121)	1 in 336 (269-429)[e]
	NNP_2-SRBC	1 in 90 (86-94)	1 in 87 (84-91)[c]	nd	nd
	$NNP_{0.2}$-SRBC	1 in 503(459-552)	1 in 449(380-544)[c]	1 in 516 (392-678)	1 in 837 (633-1072)[c]
	TNP_{30}-SRBC	1 in 96 (85-106)	1 in 93 (83-103)[c]	1 in 162 (112-234)	1 in 201 (154-263)[c]
BM	NIP_4-SRBC	1 in 24 (20-29)	1 in 40 (38-42)[f]	nd	nd
	$NIP_{0.4}$-SRBC	1 in 211(181-246)	1 in 270(242-300)[c]	nd	nd
	NNP_2-SRBC	1 in 99 (84-117)	1 in 171(145-202)[f]	nd	nd
	$NNP_{0.2}$-SRBC	1 in 587(439-784)	1 in 570(518-627)[c]	nd	nd
	TNP_{30}-SRBC	1 in 151(136-167)	1 in 325(278-380)[g]	nd	nd

a. Figures represent the mean ratio of specific IgM-antibody secreting cells to the total number of IgM-secreting cells as determined in the protein A plaque assay. The geometric mean $\pm$ 1 SE has been calculated. In the spleen of 8-12-week-old CV-NI and GF-CD mice (n=20) and the spleen of 52-week-old CV-NI and GF-CD mice (n=10), 3950 (3850-4050), 3715 (3519-3928), 2668 (2312-3079) and 4441 (4115-4792) IgM-secreting cells per 10^6 nucleated cells were found, respectively. In the BM of 8-12-week-old CV-NI and GF-CD mice (n=8), 451 (439-461) and 621 (605-632) IgM-secreting cells per 10^6 nucleated cells were found, respectively.

b. For statistical evaluation the data of the GF-CD mice were compared with those of the age-matched CV-NI controls.

c. Not significantly different.

d. nd = Not determined.

e. $P < 0.01$.

f. $P < 0.05$.

g. $P < 0.001$.

that revealed MLN, the figures were 4, 25 and 30 times lower, respectively.

3.2 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 of 8-12-week-old and 52-week-old CV-NI and GF-CD mice and in the BM of 8-12-week-old CV-NI and GF-CD mice by employing the protein A plaque assay and antigen-specific plaque assays with the use of a panel of five different types of target cells. The results given in Table 2 show that the frequency of IgM-secreting cells specific for the various haptenized SRBC tested in the spleen of 8-12-week-old CV-NI and GF-CD mice did not differ. In the spleen of 52-week-old GF-CD mice the cells secreting antibodies of the specificities tested tended to be somewhat less frequent than in the spleen of their age-matched CV-NI controls, the latter matching the frequencies of the 8-12-week controls. In the older animals of both groups more variable data were obtained as can be deduced from the standard errors of the mean. In the BM of the CV-NI and GF-CD mice, the frequencies of IgM-secreting cells specific for the antigens tested were much alike and when compared with the data obtained in spleen no striking differences were observed except for TNP_{30}-SRBC. Thus in the antigen-free mice and the conventional controls a similar proportion of the IgM plaque forming cells secretes antibodies of the defined specificities.

4. DISCUSSION

The aim of this study was to establish the role that exogenous antigens and/or mitogens might play in the development of the B cell specificity repertoire as it is expressed by 'spontaneously' occurring ('background') Ig-secreting cells in unprimed mice. Such studies are now feasible with the development of special diets and improved housing and breeding conditions for 'antigen-free' mice (2,3). From our data it can be concluded that as far as the pool of IgM-secreting cells is concerned no role is reserved for exogenous stimuli to affect the antibody repertoire that these cells express as tested with our panel of antigens. This suggests that available antibody repertoires are established independently of exogenous antigenic or mitogenic stimulation. Therefore endogenous stimulation must account for the activation of background Ig-secreting cells and in general for the selection of particular repertoires of B lymphocytes. It can be speculated then that these endogenous stimuli are provided by idiotypic determinants, e.g., from maternal Ig transferred to the pre- or neonates via the placenta or milk (12-14) or from any other self antigens (15,16) that either directly activate B cells or indirectly by first activating helper T cells specific for B cell membrane determinants, such as antibody idiotypes (17). Thus the basis for the intrinsic activity of the immune system is found in the individual itself. This is in direct support of Jerne's network ideas (18) postulating autonomy and completeness in a system embodied by the interaction between complementary molecules (19) including antibodies, T cell receptors and receptors controlling activation from the resting state in mature B lymphocytes and their growth and differentiation as precursors (20). It is evident from our results that young and old antigen-free mice develop Ig-secreting cells and exhibit IgM antibody specificity repertoires which are not different from those expressed in their conventional counterparts, which are surrounded, invaded and stimulated by non-self antigens and mitogens.

Our data concerning the number and distribution of Ig-secreting cells of the various isotypes in antigen-free mice fit into the earlier formulated concept that the background Ig synthesis in the BM and MLN, especially the IgG and IgA synthesis, is mainly dependent on exogenous antigenic stimulation, whereas the background Ig-synthesis in the spleen is mainly due to endogenous stimulation (5). As was pointed out by Koch et al. (21) and reviewed by Benner et al. (22), antibody-secreting cells in the BM are derived from B memory cells, reactivated by antigen in the peripheral lymphoid organs. Thus, prolonged low-level endogenous stimulation might, in the end, induce the generation of B memory cells, reactivate them and thereby induce Ig synthesis in the BM. This can explain the difference in numbers of IgG-secreting cells between the BM of conventional and antigen-free mice and the age-related increase of the number of Ig-secreting cells in the BM of GF-CD mice.

The effect of exogenous antigenic stimulation upon the gut-associated lymphoid tissue is most dramatically shown by GF-CD mice. In 50% of the young GF-CD mice and in 90% of the 52-week-old GF-CD group no MLN could be detected, in contrast to the CV-NI controls. Thus, the lack of antigenic stimulation by food and bacteria in the gut diminishes the size of the MLN and reduces the number of Ig-secreting cells in this organ. Furthermore, it must be noted that the low numbers of IgG- and IgA-secreting cells fully explain the data obtained in serum analyses of GF-CD mice: low levels or absence of IgG and no IgA (4,6).

The data presented in this report reflect the end stage of the development of the B cell repertoire as it has developed in mice completely devoid of exogenous antigenic stimulation. It remains to be established, however, if such animals are still equally capable of reacting to exogenous antigenic and mitogenic stimuli as conventional mice. Studies to analyze this aspect as well as the antibody-specificity repertoire of the mitogen-reactive B cells are in progress.

ACKNOWLEDGMENTS

We thank Ms. T. Hooijkaas-van Leeuwen and Ms. A.A. van der Linde-Preesman for skilful technical assistance, Ms. C.J.M. Meijerink-Clerkx for typing the manuscript and all members of the Lobund Laboratory who in some way contributed to this work.

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CHAPTER XII

GENERAL DISCUSSION

In the foregoing chapters the process of B cell differentiation has been studied at the cellular level in an attempt to gain more insight into the regulatory mechanisms involved in the generation, selection and expression of the B cell repertoire including the Ig isotypes. To this end, LPS-reactive B cells and 'spontaneously' occurring ('background') Ig-secreting cells from young and aged conventional thymus-bearing and T cell-deficient mice were analyzed (Chapters VII-IX). The same was done with thymus-bearing 'antigen-free' mice (Chapters X and XI). An optimized *in vitro* limiting dilution mitogen assay (Chapters IV and V) allowed frequency determinations of clones producing the various Ig isotypes (C gene expression) and of clones producing specific antibodies (V gene expression). Specificity analyses of individual background Ig-secreting cells, representing the terminus of B cell differentiation, provided information on the actual B cell repertoire. In general, it can be concluded that the available B cell repertoire is the same in young and old conventional thymus-bearing and T cell-deficient mice as well as in thymus-bearing 'antigen-free' mice (Chapters VII-X). The actual repertoire, on the other hand, proved to be quantitatively and/or qualitatively different under these circumstances (Chapters VIII, IX and XI). This indicates that the available repertoire can be established independently of exogenous antigenic and/or mitogenic stimulation and the presence of T cells, suggesting that endogenous stimuli account for it. Thus, the generation of antibody diversity is an antigen-independent process. Studies of pre-B cells revealed that a putative selection of B cell repertoires might operate at or before the pre-B cell differentiation stage (Chapter VI). Alternatively, it can be hypothesized that no selection at all is involved in the establishment of the available repertoire and that it essentially represents the germline repertoire. However, since the actual antibody repertoire, being selected from the available one, does show differences in expression, it might be suggested that selection mechanisms, be it exogenous antigen or the idiotypic network, are at work at the level of immunocompetent B cells when they enter the (long-lived) peripheral lymphoid compartment.

Some additional remarks will be made concerning the general validity of the aforementioned results obtained with the assay systems employed. Finally we will evaluate whether they fit in existing ideas about the function of the immune system and in explanations of the mechanisms that might operate in it.

It must be remembered that all data obtained with the LPS limiting dilution culture assay relate to about one third of the newly-formed B cells. Thus, LPS does not activate all B cells but only a subset. It is generally thought that these LPS-reactive B cells are representative for the B cell specificity repertoire since polyclonal activation does not involve the variable region of Ig, providing a basis for random activation of antigen-specific LPS-reactive B cells. It has, however, been shown that the idiotypic repertoire is not equally distributed among B cell subpopulations as defined by mitogen reactivity (Primi et al., 1982a, b). This could mean that a specific antibody could be absent in the LPS-activated repertoire. However, since in the studies performed in this thesis the repertoires of LPS-activated B cells from different groups of mice have been compared and no major differences have been found, this objection would not apply. One of the ways

to overcome a restricted LPS-response would be the use of mixtures of mitogens such as LPS and *Nocardia* water-soluble mitogen (Bona et al., 1978), LPS and carrageenan (Kolb et al., 1981) or LPS and dextran sulfate (Kettman and Wetzel, 1980; Wetzel and Kettman, 1981a, b; Bergstedt-Lindqvist et al., 1982) to stimulate several complementary mitogen-reactive subsets of B cells for repertoire analysis. The use of various mitogens may also define subsets of B cells with different reactivities. Combining these approaches and improving (serum-free) culture conditions for B cell growth and differentiation (Corbel and Melchers, 1984; Melchers et al., 1984; Zubler, 1984) a larger proportion of B cells, if not all, would be analyzable. However, data over repertoire analysis obtained with such well-defined systems have not yet been reported.

It is not known in what manner the Ig isotype switch is induced or regulated. Opinions on the possible regulation mechanisms differ. Briefly, two theories are favored. According to the multilineage model, each individual B cell is precommitted to the production of a particular isotype (Abney et al., 1978). Thus, B cell development is accompanied by isotype commitment in the absence of any extrinsic influences, such as antigens or T cells (Lawton and Cooper, 1974; Lawton et al., 1975; Calvert et al., 1983). The single lineage model, on the other hand, states that the B cells are multipotential with respect to the isotype production of their progeny. Experiments using clonal assay systems demonstrated that a single B cell, stimulated by antigen or mitogen, can give rise to progeny that produces antibodies with isotypes other than IgM (Press and Klinman, 1973; Gearhart et al., 1975, 1977, 1981; Andersson et al., 1978; Ventura et al., 1978; Wabl et al., 1978; Van der Loo et al., 1979). The results described in Chapter VII confirm these data for splenic B cells and also show them to be true for the B cells present in BM, MLN and thoracic duct. Single B cells can undergo more than one switch event (Gearhart et al., 1980, 1981; Teale et al., 1981; Coutinho and Forni, 1982), although there is also evidence that the major switch pathways are directed from cμ to each of the other isotypes (Webb et al., 1983) or at least from cμ to IgA or IgE (Coutinho and Forni, 1982). In *in vitro* studies with human peripheral blood (Kuritani and Cooper, 1982) or tonsils (Mudde et al., 1984) no evidence for multiple switch events has been found. Moreover, with the use of PWM or EBV for the *in vitro* stimulation of peripheral blood B cells under clonal conditions, no switching from one to another isotype was observed (Stevens et al., 1981; Yarchoan et al., 1983). This contrasting result does not necessarily indicate differences between murine and human B cells, but might just as well suggest that different B cell subsets express different functional potentials *in vitro*, the human PWM or EBV activated subset being more mature than murine LPS-reactive B cells and therefore already having undergone isotype switching *in vivo*.

In particular, the data obtained with preselected B cells of a particular isotype (Gearhart et al., 1981; Teale et al., 1981) suggest that B cells switch their isotypes at the DNA level in a 5'- to 3'-direction, thus consistent with the germ line order. However, some data have been reported that switches in the opposite direction ('back-switch') can also occur (Ventura et al., 1978; Davis et al., 1980; Radbruch et al., 1980), although this may not have a physiological meaning (Sablitzky et al., 1982). Furthermore, it was shown that in the mouse system certain stimuli such as thymus (in)dependent antigens, mitogens, T cells or T cell lines and their secreted soluble products, in many, but not all, cases (Pierce et al., 1978; Teale, 1983a) preferentially give rise to the production of a particular isotype. Thus, IgG3

and to a lesser extent IgG2b, appear to be 'thymus-independent' isotypes, whereas IgG1, IgG2a, IgE and IgA can be classified as 'thymus-dependent' ones (Coutinho et al., 1982; Rosenberg, 1982; Bergstedt-Lindqvist et al., 1984; Vitetta et al., 1984; Chapters VII and IX). Moreover, the observed abnormalities in isotype expression in the partially B cell deficient CBA/N mouse could not be attributed to an intrinsic B cell defect (Teale, 1983b). These data have mostly been interpreted that T cells direct the isotype switching process, although it can not be excluded that T cells simply select isotype-committed B cells for preferential induction of terminal differentiation, since most of the data - including those described in this thesis - are based on measurements of antibody secreting cells or secreted antibody. This might be insufficient when considering C_H gene expression, since it has been found that LPS-activated B cells may switch to IgG1 and express this isotype as a membrane-bound Ig but do not secrete it (Forni and Coutinho, 1982). It was suggested (Coutinho et al., 1983), that helper T cells regulate via functionally selective B cell specific factors the IgG1 expression by two alternative mechanisms, which lead either to proliferation of IgG1-bearing cells or to the terminal maturation of cells secreting IgG1 at a high rate. Also in the human system T cells can preferentially influence the terminal differentiation of B cells (Mayumi et al., 1983). In both cases none of the helper T cell activities appear to *induce* switching events, suggesting that T cells are not a prerequisite for the switching event *per se* (Abney et al., 1978; Calvert et al., 1983). This is, however, in contrast to a recent report which indicates that T cells directly induce B cells to switch to IgA (Kawanishi et al., 1983).

The role of exogenous antigens and/or mitogens in the generation of isotype diversity is also still controversial. They have been used in most of the aforementioned experiments to stimulate the B cells, but germfree (GF) mice also developed different classes of B lymphocytes as measured by surface Ig expression (Lawton and Cooper, 1974; Lawton et al., 1975). It is, however, clear that in GF mice the serum IgG and IgA levels are low or even undetectable (Wostmann et al., 1971; Hashimoto, 1978), while the numbers of 'spontaneously' occurring ('background') IgG and IgA secreting cells are severely diminished (Chapter XI). This suggests that exogenous antigens and T cells exert a profound influence on the generation of cells that *secrete* these Ig classes.

It has been postulated that randomness in the genetic events involved in isotype switch, occurring with decreasing probability with increasing distance from the gene segment coding for the constant region of IgM, would dictate the frequency in which the isotypes are expressed (Gearhart et al., 1980), and some data do fit in such a model (Mongini et al., 1982, 1983; Chapter VII). As a consequence, cells belonging to a clonal progeny, will successively delete C_H genes and express those located most 3' on the chromosome, finally ending up with IgA (Gearhart et al., 1980; Gearhart and Cebra, 1981). However, this model has not been confirmed in *in vivo* (Björklund and Coutinho, 1982, 1983) and *in vitro* experiments (Bergstedt-Lindqvist et al., 1982), since there was no preferential increase in one isotype at the expense of another. Much of the data suggest that the isotype switch event is regulated by still unknown C-gene specific mechanisms. These might be activated by signals that also trigger B lymphocytes to growth and maturation (Coutinho et al., 1982; Möller et al., 1983), phenomena that occur both in ontogeny and in the development of antibody responses.

The present results, like all analyses of diversity, are necessarily restricted to a small fraction of the specificity repertoire and the specificity of the antibodies is operationally defined by the indicator antigens used. Therefore, an antibody is called specific if it gives hemolytic plaques in the direct Jerne plaque assay. However, cells lysing their target antigens can do this with different affinities ranging from high to low (just detectble as PFC). Moreover, it has been found that certain antibodies elicited by a particular hapten can bind with even greater affinity to a different hapten. These antibodies were called heteroclitic antibodies (Mäkelä, 1965; Walters and Wigzell, 1972). In addition, a single antibody molecule has been found to possess several different combining sites, which means that it is in principle multispecific (Richards and Konigsberg, 1973; Varga et al., 1973; Richards et al., 1975; Cameron and Erlanger, 1977; Atassi, 1980; Jerne, 1984). Therefore, it should be kept in mind that specificity for haptens or heterologous erythrocytes represents a specificity pattern of crossreactive multispecific antibodies. Thus, any one antibody of the repertoire can react with more than one antigenic determinant and any one antigenic determinant can react with more than one antibody. This implies that the immune system is degenerate and redundant, consisting of a repertoire of overlapping recognition units which, however, in its entirety can effectively bind to any antigen. To obtain more information about the fine specificity of the antibodies produced, anti-idiotypic reagents should be used as discussed in Chapter X, but these have not been raised for C3H/HeCr mice. Therefore, GF BALB/c mice have been reared on an 'antigen-free' diet to analyze B cell precursors specific for certain idiotypes which can be detected with available anti-idiotypic antibodies. It can, however, be envisaged that in the end examination of V region-coding DNA of hybridomas constructed with B cells of relevant mice might enable a more in-depth analysis of the B cell repertoire.

In vitro studies of the B cell repertoire of BM pre-B cells and spleen cells of thymus-bearing and nude mice, employing the LPS limiting dilution culture system and anti-idiotypic antibodies (Juy et al., 1983; Nishikawa et al., 1983), revealed similar conclusions as have been derived in this thesis: neither influence of T cells on repertoire generation nor selection of the repertoire during the differentiation of BM pre-B cells into peripheral LPS-reactive B cells. This leads to a point of criticism made by Jerne (1984) about the value of repertoire analyses by LPS activation of newly-formed B cells. This aspect needs further clarification and discussion, which should lead to a general concept of the generation and maintenance of the B cell repertoire.

The mouse BM has been shown to produce 13 to 17 x 10^6 B cells per day (Opstelten and Osmond, 1983; Fulop et al., 1983). It was suggested that the usual rate of B lymphocyte production is determined by short-range microenvironmental factors and inductive interactions with stromal cells (Osmond and Batten, 1984) although systemic administration of a variety of potentially antigenic and non-antigenic agents can stimulate the BM lymphocyte production (Osmond et al., 1981; Fulop and Osmond, 1983). This would imply that B cell genesis is normally amplified by environmental stimuli, probably a polyclonal proliferative effect mediated by macrophages (Fulop and Osmond, 1983). In spite of this daily production of at least 10 x 10^6 B cells, the total number of mouse B cells remains virtually constant. This implies that the great majority of these newly arising B cells must die, either in the BM or in the peripheral lymphoid organs (de Freitas and Coutinho, 1981). Until now a satisfactory explanation for this high turnover has not been given.

This massive production and decay of B cells, analogous to the apparent production and elimination of immature T cells in the thymus (Scollay et al., 1980), might have something to do with selection mechanisms that enable the maintenance of B cells with the required specificity. As discussed above, it is generally thought that receptor structures arise randomly in precursor cells by DNA rearrangements independent of antigens in the environment of the cells. When cells express these receptors on their surface membrane they are on principle targets for selection. This does not imply that only cells expressing sIg can be such targets, since it was shown that growth receptors on sIg^- precursor B cells have determinants crossreactive with germline V gene products (Forni et al., 1979; Coutinho, 1980). These receptors could provide a way for the selection of the desired idiotypic specificities at this differentiation stage. In this respect it is relevant that the large pre-B cells in the BM produce twice the number of cells needed to maintain the small pre-B cell population (Landreth et al., 1981; Opstelten and Osmond, 1983). On the other hand, data provided in Chapter VI and by others (Nishikawa et al., 1983; Juy et al., 1983) do not indicate that such a specific selection takes place during the transition of large to small pre-B cells and subsequently to LPS-reactive B cells. Nevertheless it has been shown *in vitro* and *in vivo* that in principle, selection can occur early in differentiation via anti-idiotypic antibody. If anti-idiotypic antibody is present in cultures during the differentiation from the pre-B to the B cell stage, LPS-reactive B cells expressing the complementary idiotype do not appear (Nishikawa et al., 1983). Administration of anti-idiotypic antibodies to mice, suppressed idiotype expression as revealed upon immunization (Kelsoe et al., 1980, 1981; Reth et al., 1981; Takemori and Rajewsky, 1984a, b) and by lower LPS-reactive precursor frequencies specific for that idiotype (Bernabé et al., 1981a; 1981b).

Jerne (1984) has made an attempt at the bookkeeping of the turnover of lymphocytes and Ig molecules in a normal mouse by constructing a model of an imaginary mouse of 10 weeks old. Some features of this mouse, as they are relevant for this discussion, will be described. Excluding the cells in its thymus and BM, the imaginary mouse possesses 2×10^8 T cells and 10^8 B cells. About half of the B cells is short-lived and apart from a small fraction ($\pm$ 5%) they die exponentially with a halflife of 2.5 days. The other 5×10^7 B cells are long-lived stable cells. For unclarified reasons, about once every second a B cell from this stable pool is activated, proliferates and forms a small clone of Ig-secreting cells. On average, this clone reaches a maximal size of about 75 cells in 5 days, then rapidly dying away. Thus about 10^5 such incipient clones may arise every day, and the sum of these clones can account for the 10^6 background Ig-secreting cells found to be present in a 10 week old mouse (Benner et al., 1982). About 1 in 10^5 of the stable B cells produces Ig that may react with SRBC, which means that about 1 such B cell is activated every day. As this is a Poisson process, the actual number can vary every day, explaining the large differences that can be obtained in the number of specific background PFC against SRBC in different mice from the same source. On the other hand, it has been argued (Jerne et al., 1974) that about 15 B cells specific for SRBC are present in the presumably 5% short-lived cells (1.25×10^6) that enter the pool of stable cells, but not yet the recirculating pool. These 15 B cells (their actual number can vary greatly among individual animals) are thought to undergo clonal expansion upon immunization with SRBC. On the other hand, in the spleen, about 10^4 B cells are present that can form antibodies against SRBC after stimulation with LPS (Andersson et al., 1977; Chapters VIII and X). However, these cells are not

likely to be involved in the SRBC response, as this is thought to be mediated by long-lived cells (Jerne, 1984; Van Oudenaren et al., 1983, personal communication), and since treatment with LPS *in vivo* as well as *in vitro* almost only stimulates the short-lived lymphocytes, which the imaginary mouse never calls upon, except when invaded by mitogen-carrying organisms. Therefore, it would not be surprising to find such similarities in the available B cell repertoire among the mice tested in the LPS-mitogen assay, since this available repertoire truly reflects the B cell repertoire of newly-formed B cells, from which only a small proportion is allowed to enter the pool of stable B cells. What mechanism determines which cells are permitted to make this transition is, however, unknown. It seems clear that this mechanism can not be revealed by analyzing the LPS repertoire only. Therefore, attempts should be made to compare it with analyses of the stable pool of antigen-reactive B cells as reflected by memory B cells or the 'spontaneously' occurring ('background') Ig-secreting cells, the latter also analyzed in this thesis. Maybe repertoires analyzed in the splenic focus assay, which is thought to activate, together with primary B cells, the long-lived B cells, is also suitable. Once a stable pool of B cells and Ig has been established (and the rules for this process are not known although a role for maternal influences can be envisaged, see later) and since only 10^5 B cells disappear from the stable pool per day, it follows that the immune system of an adult animal (apart from its short-lived elements) possesses stable repertoires (Jerne, 1984). The rapid disappearance of antibody molecules from the blood after an immune response, can then be viewed upon as the rejection of these Ig structures to which the stable system allows only short lifespans. Therefore, a central feature of the immune system is the establishment of a stable idiotypic network which is based on self and anti-self and which declines in stability with age (Jerne, 1984).

The problem of self-nonself discrimination has been made a central issue in the immune system and it has become clear that failure to react to autologous antigens is not due to a single causative mechanism but rather is the end result of a number of regulatory processes that mutually reinforce each other and have been called 'tolerance induction', 'clonal anergy' and 'antigen-receptor blockade' (Nossal, 1984). However, it appears important that tolerance should always be thought of in quantitative rather than in absolute terms, since it has been argued (Grossman, 1984), that the distinction between self and non-self might be determined by the balance between self-renewal and differentiation to effector function of lymphocytes (balance of growth hypothesis formulated by Grossman, 1982). Recognition with high affinities of abundant self leads to enhanced differentiation with, as a consequence, functional elimination of these self-reacting lymphocytes. On the other hand, self-recognizing clones with a certain range of low affinities below the terminal differentiation threshold, proliferate and become prominent. Thus, the amount of (self) antigens and the nature of encounter with lymphocytes (sudden, graded or continuous) can be correlated with a weak or a strong expression of effector function and therefore with the generation of effective memory or of tolerance (Grossman, 1984). Some degree of autoantibody production is probably perfectly normal and has actually been demonstrated in the serum (Guilbert et al., 1982; Avrameas et al., 1983; Hijmans et al., 1984). Moreover, hybridomas producing antibodies against self constituents have been constructed with spleen cells of healthy mice (Dighiero et al., 1983; Holmberg et al., 1984; Prabhakar et al., 1984; Wassmer, 1984), thereby disproving the idea that autoreactive clones would be eliminated (clonal abortion), but rather confirming that the actual antibody repertoire

is complete.

Another model that might contribute to the mechanism of self-nonself discrimination has been proposed by Jerne (1984). It states that in ontogeny self-antigens will induce the production of anti-self Ig molecules. These, binding to self, then invoke the production of anti-idiotypic molecules. These in turn can prevent the future expression of the corresponding idiotypes. Normal Ig (natural antibodies) are therefore thought to consist of a mainly stable population of molecules that are anti-idiotypic to the antiself molecules. Viruses and bacteria that multiply in the body can not escape because of the ever renewed complete repertoire. It can be speculated that invaders mimicking 'self' are hard to deal with by the immune system because they evoke a continuous formation of anti-idiotypic antibodies to the antibodies directed against them. However, extensive genetic polymorphism of cell membrane antigens encoded for by the major histocompatibility complex (MHC) occurs in all mammalian species studied, so that a population that is periodically confronted with different pathogens can escape from total extinction.

From the foregoing, a picture emerges in which the immune system is predominantly occupied in defending the body against external threats. However, some other points of view concerning the function of the immune system also deserve attention, especially since our experiments with the 'antigen-free' mice are relevant in this respect (Chapters X and XI). This will be discussed in the following paragraphs.

Classical immunology is based upon the notion of immune responses against foreign materials and absence of autoreactivity. This requires the definition of 'foreigness' and consequently the discrimination between 'self' and 'nonself'. Another component in the classical view is the notion that the immune system is devoid of activity in the absence of those foreign materials that induce immune responses. Antibodies should only exist as the consequence of the presence of (foreign) antigens. However, it has been shown that the immune system is characterized by an internal activity which is generated in the absence of exogenous antigens (Chapters X and XI). This autonomous activity may be referred to as the immunological self (Coutinho et al., 1984) and it is even quantitatively far more important than the immune responses to foreign antigens. These may be described as disturbances of the internal activity which require compensation. All lymphocytes play the exclusive function of maintaining reference levels for self: antibodies and effector cells are complementary to internal structures and only bind foreign materials as 'external images' of these internal targets. Such a system only knows of itself and its' self is the network of endogenous activity.

As argued by Coutinho et al. (1984), immunology has started by being a subsidiary discipline almost exclusively concerned with the production of 'good antisera' to be used in diagnostics or therapeutics. Therefore, the attainment of the teleological conclusion that the purpose of the immune system was to defend the organism against infectious agents became inevitable. However, it has been advocated that the immune system is more than 'a sophisticated biological warfare machine' and it has been proposed that immunology may be regarded not as antigen centered but as organism centered (Vaz and Varela, 1978; Varela, 1979; Coutinho et al., 1984). The immune system should then be regarded as the essential regulator of the molecular identity of the individual, embodied by processes of cooperative interactions. Until now, most forms of immunological experimentation bear little relationship to the

types of antigenic stimulation met in the normal ecological environment of the individual. Immunological reactions are induced by artificial routes (intraperitoneally or intravenously), frequently utilizing so-called adjuvants which may boost the responses to abnormally high levels. It might be that these methods induce major disturbances in certain parts of the immunological network. In contrast, the antigenic stimulation by oral or respiratory exposure to foreign antigens leads to different, quite opposite, reactions (Vaz et al., 1977; Kagnoff, 1982; Challacombe, 1983). Therefore, it has been argued that clues concerning the normal operation of the immune system have been searched by creating rather abnormal conditions of stimulation (Coutinho et al., 1984). It might therefore be far more opportune to study the normal immune system with as little intervention as possible and to describe its structures and internal activity in an unmanipulated situation and to describe interactions among its elements and of these with other components in the organism. It has been argued (Vaz et al., 1984) that the immune system might participate in the modulation of all other molecular interactions in the organism, e.g. those involved in the neuroendocrine system. Consequently, autoreactivity can then be considered as normal. Such physiologic immune autoreactivity does not lead to destruction of self targets, because it does not represent immune responses but the manifestation of equilibria within a network of autonomous activities. Antiself activity could be neutralized by anti-idiotypes to the auto-antibodies present in the actual repertoire. The results obtained with 'antigen-free' mice (Chapters X and XI) would fit in this concept of an immune system not primarily occupied with the fighting against microorganisms, but maintaining reference levels for self, that are internally generated in the absence of foreign antigens. All actions of the system are aimed at the maintenance of these reference levels (its organization) through modifications of its composition (structure), in response to internal and (not necessarily) external perturbations (Vaz and Varela, 1978; Varela, 1979; Vaz et al., 1984).

Since only the IgM antibody repertoire has been studied (Chapters VI, VIII-XI), it is pertinent to say that the available IgM antibody-specificity repertoire is neither affected by the process of aging, nor by the presence of T cells or exogenous antigens. No statements can be made about the IgG or IgA antibody-specificity repertoire, yet. It is obvious that the immune network can (sufficiently?) exist of only IgM antibodies since in 'antigen-free' mice the serum IgM level is only moderately decreased while IgG and IgA are hardly detectable in the serum. This may suggest that the network of Ig primarily exists of IgM and that IgG and IgA are not necessarily involved, although they can of course easily be incorporated via their V-regions. In addition, the absence of T cells does not prevent the presence of IgM-secreting cells but does affect the number of cells secreting IgG or IgA, as has been shown in the experiments with T cell deficient nude mice (Chapter IX). Together with additional evidence (Etlinger and Heusser, 1983), this suggests that a functional network of idiotypic-anti-idiotypic interactions can occur in the absence of T cells. However, T cells do affect the specificity repertoire of background IgM-secreting cells (Chapter IX). This might be explained by supposing that T cells via their T cell receptor repertoire contribute to the selection of actual B cell repertoires. Similarly, differences have been reported in the repertoire of helper T cells in normal and agammaglobulinemic mice (Coutinho et al., 1984). Thus, the repertoire of T cells can be influenced by B cells, presumably via their surface Ig receptors or secreted Ig, while the repertoire of B cells can be influenced by T cells, presumably via their antigen receptors.

An important issue concerning the ontogeny of the B cell repertoire or network has not been explicitly addressed in this thesis but needs further discussion in relation to the 'antigen-free' mice. It has been suggested that in ontogeny the immune system develops from an incipient network (Adam and Weiler, 1976; Holmberg et al., 1984) which could mean that normal Ig is a mainly stable population of molecules that are anti-idiotypic to the antiself molecules, both encoded in the germline (Jerne, 1984). This might well be so and much attention has been given to the transfer of maternal Ig to the pre- or neonates via placenta or milk, as a trigger for the development of the antibody network (Weiler et al., 1977; Weiler, 1981; Bernabé et al., 1981a, b). Thus, the basic driving forces for initiating the intrinsic activity of the immune system, apparently not delivered by microorganisms, could be exerted by idiotypes derived from maternal Ig apart of course from any other self antigens. In experimental situations, it has indeed been shown that maternal idiotypic suppression can be transferred from the mother to the offspring (Bernabé et al., 1981a, b). On the other hand, it has been shown that in GF colostrum-deprived piglets no serum Ig could be detected, probably due to the six-layered epitheliochorial placenta, impermeable to antigens and Ig (Sterzl, 1967; Kim, 1975). In addition, no background antibody-forming cells against SRBC could be detected in their lymphoid organs, although the piglets proved to be normally immunocompetent upon antigenic challenge (Sterzl, 1967; Kim, 1975). However, the spleen of pigs delivered by caesarean surgery, and sacrificed immediately after delivery, contained several thousand IgM- and IgG-secreting cells, as detected by the protein A plaque assay (Bianchi, 1984, personal communication). These results might indicate that the immune system can start its activity autonomously, but that maternal Ig can influence the quality of this network as in the newly born pig the gut tissue is extremely permeable for milk constituents which enables the transfer of Ig molecules and consequently of idiotypes. This could be relevant for the building up of the immune network of the newborn, which is adequate for the environment in which it lives. This would imply that the immune system of each individual is influenced by the immune systems of former generations.

A totally different explanation of the existence of Ig and Ig-secreting cells in even 'antigen-free' animals (Chapter X and XI) has been proposed by Grabar (1975, 1983). He considers Ig molecules as specific transporters of metabolic and catabolic products facilitating the cleansing of the organism by opsonization which favors phagocytosis. According to his view, auto-antibodies are normal constituents of serum, serving as transporters of catabolic products. Thus, Ig are not considered as a specific defense system, but a physiological mechanism for cleaning the body of unwanted material, including foreign substances as microorganisms.

The experiments described in this thesis have been performed with B cells derived from unprimed animals of various ages, some of them athymic, others kept under antigen-free conditions. For this purpose, the elaborately designed architecture of the lymphoid tissue used was destroyed in order to obtain single cell suspensions of lymphocytes. However, it seems justified to consider results obtained with cell suspensions within the anatomic and physiologic framework of the intact animal (Osmond and Batten, 1984). In particular, for the analysis of the B cell repertoire it seems sensible to isolate B cell subpopulations on, for example, morphological grounds to assess their repertoire to gain more insight into the repertoires of not only newly formed, but also recirculating memory B cells or B cells present at different anatomical sites as the gut- or bronchus-associated lymphatic tissues. *In situ* marker

analysis combined with functional assays of isolated populations of B cells or B cell cloning might prove to be fruitful approaches for unraveling the rules that govern the establishment and maintenance of B cell repertoires. It is obvious that neither DNA analysis, cellular immunology or *in vivo* immunology alone will be sufficient to explain the mechanisms governing the immune system. The combination of these three levels - subcellular, cellular and organismal - are needed to be brought more tightly together since only the *combination* of these approaches will provide the power to unravel the mechanisms at work in the immune system.

XII.1 References

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SUMMARY

The total number of different immunoglobulin (Ig) molecules that the immune system produces is often called the antibody specificity repertoire or B cell repertoire (Chapter I). This repertoire can be subdivided into three categories: the potential, the available and the actual repertoires. The potential repertoire is determined by the number, structure and mechanisms of expression of the germline genes encoding Ig molecules plus the possible somatic variants derived from them and can be regarded as what potentially can be made. The available repertoire is defined as the set of diverse antibody molecules that are expressed by immunocompetent but resting B lymphocytes and can be looked upon as what has been made and can be used. The actual repertoire is represented by that set of antibodies that is actually secreted by B cells and can be regarded as what is actually being used.

Little is known about the regulatory mechanisms that enable the establishment, from the potential repertoire, of the available and functionally expressed repertoire of the immunocompetent resting B cell compartment. Similarly, the mechanisms that govern the establishment of the actual repertoire from the available repertoire are only partly known. Therefore, the purpose of the studies presented in this thesis (as outlined in Chapter III) was to obtain more information concerning the regulatory mechanisms that are involved in the functional expression of the Ig C and V genes by murine B cells. To this end, frequency analyses of B cells secreting particular Ig heavy chain isotypes (C gene expression) and specific IgM antibodies (V gene expression) were performed among the progeny of B cells that had differentiated from pre-B cells *in vitro*. The same analyses were performed on *in vivo* generated mitogen-reactive B cells (available repertoire) and on the 'spontaneously' occurring ('background') Ig-secreting cells (actual repertoire). The possible regulating influences studied include age, T cells and exogenous antigens. The latter became feasible, since, with the successful breeding of germfree mice fed an ultrafiltered solution of chemically defined low molecular weight nutrients, exogenous stimuli such as antigens and mitogens can be reduced to a minimum never attained before.

Newly-formed B cells were studied at the clonal level in the *in vitro* limiting dilution culture assay, employing lipopolysaccharide (LPS) as a mitogen, which stimulates, depending on the mouse strain studied, about every third B cell to growth and maturation into a clone of Ig-secreting cells (Chapter II). *In vivo* 'background' Ig production was measured at the single cell level. Both the LPS-stimulated B cell clones and the 'background' Ig-secreting cells were analyzed for the isotype distribution of the Ig they produced and for specific IgM-antibody production. The cells secreting Ig of a given (sub)class were demonstrated with the protein A plaque assay and cells secreting specific IgM antibodies were determined in antigen-specific plaque assays (Chapter II). The protein A plaque assay has been improved by the use of Ig-depleted guinea pig serum as a source of complement (Chapter IV). We optimized the LPS culture system by low-dose X-irradiation (0.11 Gy) of the feeder layer of rat thymocytes to reduce the number of disturbing plaque-forming cells of rat origin in the cultures (Chapter V).

Large pre-B cells, isolated from the bone marrow (BM) by 1 g velocity sedimentation, are able to differentiate *in vitro* into LPS-reactive B cells (Chapter VI). After *in vitro* stimulation with LPS of *in vitro* and *in vivo* generated LPS-reactive B cells, both populations have an equal ability to

give rise to clones of IgM-secreting cells and to switch to other isotypes. Since also the IgM specificity repertoire of *in vitro* differentiated B cells is not different from those that have differentiated *in vivo*, it can be concluded that the antibody specificity repertoire of B cells and their capacity to switch from the secretion of IgM to the secretion of other isotypes can be established independent of *in vivo* regulatory mechanisms. This indicates that under the conditions employed there is no selection for antibody specificity during the maturation of large pre-B cells into small LPS-reactive B cells.

In the spleen, BM, mesenteric lymph nodes (MLN) and thoracic duct of BALB/c mice 5-20% of all B cells could be induced by LPS to yield a clone of IgM-secreting cells, all capable of switching to the secretion of IgG1 (4-8%), IgG2 (28-43%), IgG3 (31-49%) and IgA (1-1.5%); roughly the same frequencies of B cells switching C_H gene expression were found among splenic cells of T cell deficient athymic nude mice (Chapter VII). In addition, the antibody specificity repertoire of LPS-reactive B cells is hardly or not at all affected by T cells (Chapter IX). On the other hand, T cells do affect the isotype and antibody specificity repertoire of the background Ig-secreting cells, indicating that the actual B cell repertoire is influenced by T cells (Chapter VII and Chapter IX).

In aged mice, the proportion of LPS-reactive B cells and their repertoires are the same as in young adult mice, indicating that age does not influence the capacity of the B cell system to generate a complete, available antibody repertoire; on the other hand, the actual repertoire of the background Ig-secreting cells does change with age (Chapter VIII).

Finally, it was shown that young and old germfree mice fed an ultrafiltered solution of chemically defined low molecular weight nutrients ('antigen-free' mice), and therefore devoid of exogenous antigenic and/or mitogenic stimulation, do generate LPS-reactive B cells with a similar switching capacity and a similar IgM antibody specificity repertoire to conventionally reared and maintained mice, which are surrounded, invaded and stimulated by a variety of microbial and other non-self antigens and/or mitogens (Chapter X). Furthermore, these 'antigen-free' mice develop actual IgM antibody repertoires that are not different from those expressed in their conventional counterparts (Chapter XI). However, the number of IgG- and IgA-secreting cells in 'antigen-free' mice were significantly reduced, especially in the BM and MLN (Chapter XI).

The preceding data are discussed in Chapter XII. We conclude that the results described in this thesis indicate that the available B cell repertoire can be established independently of exogenous antigenic and/or mitogenic stimulation or the presence of T cells and is not affected by aging. This suggests that endogenous stimulation accounts for the autonomous and continuous generation of the available repertoire. A putative selection of B cell repertoires might operate at or before the pre-B cell stage. Others have hypothesized that no selection at all is involved and that the available repertoire of newly-formed B cells essentially represents the germline repertoire. However, since the actual antibody repertoire, being derived from the available one, does show differences in expression in aged and T cell deficient mice, we suggest that selection mechanisms, be it exogenous antigen and/or the idiotypic network, do influence immunocompetent B cells, when they enter the (long-lived) peripheral lymphoid compartment.

SAMENVATTING

Het totale aantal verschillende immunoglobuline (Ig) moleculen dat het immuunsysteem produceert, wordt vaak het antilichaam-specifciteitsrepertoire of B cel repertoire genoemd (Hoofdstuk I). Ten aanzien van dit repertoire kunnen drie categorieën worden onderscheiden: het potentiële, het beschikbare en het feitelijke repertoire. Het potentiële repertoire wordt bepaald door het aantal, de structuur en de expressiemechanismen van de genen die coderen voor de Ig moleculen zoals die in een bevruchte eicel aanwezig zijn plus de mogelijke somatische varianten die uit deze genen kunnen voortkomen. Het potentiële repertoire kan daarom worden beschouwd als dat wat geproduceerd kán worden. Het beschikbare repertoire wordt gedefinieerd als die antilichaam-specificiteiten die door immunocompetente maar rustende B cellen tot expressie worden gebracht, en kan worden bezien als dat wat geproduceerd is en kan worden gebruikt. Het feitelijke repertoire wordt vertegenwoordigd door de antilichamen die door B cellen worden gesecerneerd en kan worden beschouwd als dat wat feitelijk wordt gebruikt.

Er is weinig bekend over de regulatiemechanismen die, vanuit het potentiële repertoire, het beschikbare en functioneel tot expressie gebrachte repertoire van de immunocompetente rustende B cellen bepalen. Ook de mechanismen die betrokken zijn bij de totstandkoming van het feitelijke repertoire vanuit het beschikbare repertoire zijn maar ten dele bekend. Daarom was het doel van de studies die in dit proefschrift zijn beschreven (zoals uiteengezet in Hoofdstuk III) om meer informatie te verkrijgen omtrent de regulatie mechanismen die zijn betrokken bij de functionele expressie van de Ig C en V genen in de verschillende lymfoide organen van de muis. Hiertoe werden onder de nakomelingen van B cellen, die *in vitro* differentiëren uit pre-B cellen, frequentie analyses uitgevoerd van klonen van B cellen die bepaalde Ig zware keten isotypen secerneren (C gen expressie) en specifieke IgM antilichamen (V gen expressie). Dezelfde analyses werden gedaan op *in vivo* ontstane, mitogeen-reactieve B cellen (het beschikbare repertoire) en op de 'spontaan' voorkomende ('background') Ig secernerende cellen (het feitelijke repertoire) die in alle lymfoide organen worden aangetroffen. Tot de mogelijke regulerende invloeden die werden bestudeerd, behoren leeftijd, T cellen en exogene antigenen. Dat laatste was mogelijk, aangezien door het met succes fokken van kiemvrije muizen, die worden gevoed met een gefilterde oplossing van chemisch gedefinieerde voedingsstoffen met een laag molecuulgewicht, exogene prikkels, zoals antigenen en mitogenen, tot een nog niet eerder gerealiseerd minimum kunnen worden gereduceerd.

Nieuwgevormde B cellen werden op klonaal niveau bestudeerd in een limiting dilution kweeksysteem waarin LPS wordt gebruikt als mitogeen. In dit kweeksysteem wordt afhankelijk van de bestudeerde muizenstam ongeveer 1 op de 3 B cellen aangezet tot groei en uitrijping tot een kloon Ig secernerende cellen (Hoofdstuk II). De *in vivo* 'background' Ig productie werd aan individuele cellen gemeten. Zowel de door LPS gestimuleerde B cel klonen als de 'background' Ig secernerende cellen werden onderzocht op de productie distributie van de geproduceerde Ig moleculen en op specifieke IgM antilichaamproductie. Cellen die een bepaalde Ig (sub)klasse secerneren werden met behulp van de proteine A plaquetest aangetoond en cellen die specifieke IgM antilichamen secerneren met antigeen-specifieke plaquetesten (Hoofdstuk II). De proteine A plaquetest werd verbeterd door als complementbron gebruik te maken van caviaserum waaruit de Ig waren verwijderd (Hoofdstuk IV). Het LPS kweeksysteem werd geoptimaliseerd door de 'feeder layer' van ratte-thymocyten met

een lage dosis röntgenstraling (0.11 Gy) te bestralen om daardoor het aantal storende plaquevormende cellen van ratte-oorsprong in de kweken te verminderen (Hoofdstuk V).

Grote pre-B cellen, geïsoleerd uit het beenmerg door middel van 1 g snelheidssedimentatie, kunnen *in vitro* differentiëren tot kleine LPS-reactieve B cellen (Hoofdstuk VI). Na *in vitro* stimulatie met LPS van *in vitro* en *in vivo* gedifferentieerde LPS-reactieve B cellen kunnen beide populaties even goed tot klonen IgM secernerende cellen differentiëren en overschakelen naar de secretie van andere isotypen ('isotype switch'). Het IgM specificiteitsrepertoire van *in vitro* gedifferentieerde LPS-reactieve B cellen verschilt niet van dat van de LPS-reactieve B cellen die *in vivo* zijn gedifferentieerd. Hieruit kan worden geconcludeerd dat het antilichaam-specificiteitsrepertoire van B cellen en hun vermogen om over te schakelen van IgM-secretie naar de secretie van andere isotypen onafhankelijk van *in vivo* regulatiemechanismen tot stand kan komen. Dit wijst erop dat onder de gebruikte omstandigheden geen selectie optreedt voor bepaalde antilichaamspecificiteiten tijdens de uitrijping van grote pre-B cellen tot kleine LPS-reactieve B cellen.

In de milt, het beenmerg, de mesenteriale lymfklieren en de *ductus thoracicus* van BALB/c muizen kon 5-20% van alle B cellen door LPS worden gestimuleerd om uit te groeien tot een kloon IgM secernerende cellen die alle in staat waren over te schakelen op de secretie van IgG1 (4-8%), IgG2 (28-43%), IgG3 (31-49%) en IgA (1-1.5%); ongeveer dezelfde frequenties van B cellen die kunnen overschakelen op de secretie van een ander Ig isotype dan IgM werden aangetroffen in de milt van T cel-deficiënte, thymusloze, naakte muizen (Hoofdstuk VII). Ook het ontstaan van het antilichaam-specificiteitsrepertoire van de LPS-reactieve B cellen wordt niet of nauwelijks beïnvloed door T cellen (Hoofdstuk IX). Anderzijds beïnvloeden T cellen wel het isotype en het antilichaam-specificiteitsrepertoire van de 'background' Ig-secernerende cellen. Dit toont aan dat het feitelijke B cel repertoire wel door T cellen wordt beïnvloed (Hoofdstuk VII en Hoofdstuk IX).

In oude muizen zijn de frequentie en het repertoire van LPS-reactieve B cellen hetzelfde als in jong-volwassen muizen (Hoofdstuk VIII). Dit geeft aan dat leeftijd niet van invloed is op het vermogen van het B cel systeem om een compleet, beschikbaar antilichaamrepertoire te maken. Het feitelijke repertoire van de 'background' Ig secernerende cellen verandert daarentegen wel met de leeftijd (Hoofdstuk VIII).

Tenslotte werd aangetoond dat jonge en oude 'antigeenvrije' muizen, hoewel verstoken van exogene antigenen en mitogene stimulatie, LPS-reactieve B cellen produceren met een gelijk vermogen om van isotype te veranderen en een zelfde IgM antilichaam-specificiteitsrepertoire als conventioneel gefokte en gehouden muizen, die worden omgeven, gekoloniseerd en gestimuleerd door tal van microbiële niet-eigen antigenen en mitogenen (Hoofdstuk X). Bovendien ontwikkelen deze 'antigeenvrije' muizen een 'background' IgM antilichaamrepertoire dat niet verschilt van hetgeen in hun conventionele tegenhangers tot expressie komt (Hoofdstuk XI). In 'antigeenvrije' muizen was het aantal IgG en IgA secernerende cellen echter significant lager, vooral in het beenmerg en de mesenteriale lymfklieren (Hoofdstuk XI).

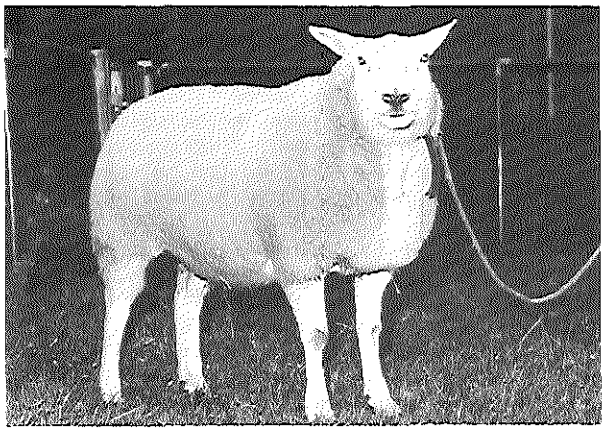
Bovenstaande gegevens worden bediscussieerd in Hoofdstuk XII. Wij concluderen uit de in dit proefschrift beschreven resultaten dat het beschikbare B cel repertoire onafhankelijk van exogene antigene en mitogene stimu-

latie en de aanwezigheid van T cellen tot stand kan komen en dat dit proces niet door het ouder worden wordt beïnvloed. Dit suggereert dat endogene stimulatie verantwoordelijk is voor het autonome en continue ontstaan van het beschikbare repertoire. Een veronderstelde selectie van B cel repertoires zou kunnen plaatsvinden tijdens of voor het pre-B cel stadium. Maar een andere hypothese zou kunnen zijn dat er helemaal geen selectie optreedt en dat het beschikbare repertoire van de nieuw-gevormde B cellen in feite het repertoire weergeeft, zoals dat in de bevruchte eicel aanwezig was. Aangezien echter het feitelijke repertoire aan antilichamen, dat ontstaat uit het beschikbare repertoire, wel verschillen vertoont in expressie in oude en T cel deficiënte muizen kan de suggestie worden gedaan dat selectiemechanismen, hetzij exogene antigenen en/of het idiotype netwerk, werkzaam zijn op het niveau van de immunocompetente B cellen, wanneer deze het (langlevende) perifere lymfoide compartiment binnengaan.

DANKWOORD

Op deze plaats wil ik graag allen bedanken die op enigerlei wijze een bijdrage hebben geleverd aan het tot stand komen van dit proefschrift.
In de eerste plaats gaat mijn dank uit naar mijn promotoren Prof.Dr. R. Benner en Prof.Dr. O. Vos voor de mogelijkheden die zij mij hebben geboden om het in dit proefschrift beschreven onderzoek te kunnen uitvoeren.
Prof.Dr. R. Benner, beste Rob, het aanstekelijke enthousiasme waarmee jij in 1979 uit het Basel Institute for Immunology bent teruggekomen, heeft het onderzoek van meet af aan in het goede spoor gebracht. Ik ben je bijzonder erkentelijk voor je gedegen begeleiding, je betrokkenheid bij het onderwerp en bovenal je stimulerende invloed, hetgeen zeer bevorderlijk is geweest voor het welslagen van het onderzoek en het afronden van dit proefschrift.
Naast Prof.Dr. O. Vos wil ik Prof.Dr. R.E. Ballieux en Prof.Dr. Th. van Joost bedanken voor de nauwgezette wijze waarop zij het manuscript hebben doorgenomen en voor de waardevolle adviezen en suggesties die zij daarbij hebben gegeven.
I want to express my special gratitude to Dr. B.S. Wostmann, Dr. J.R. Pleasants, Dr. M. Pollard and all other members of the Lobund Laboratory, University of Notre Dame, Notre Dame, Indiana, USA. It was a great pleasure to be with you, inside and outside the laboratory, and to perform experiments with your 'antigen-free' mice. The results that are described in Chapter X and Chapter XI have contributed much to the final conclusions drawn in this thesis. Furthermore, I would like to mention Dr. N.K. Jerne and Dr. A. Coutinho whose ideas have deeply influenced my view upon immunology. I thank Dr. C. Martinez-Alonso who kindly and efficiently taught me the secrets of the hapten-specific plaque assays.
Veel van de resultaten beschreven in dit proefschrift hadden niet tot stand kunnen komen zonder de betrouwbare inzet en geduldige toewijding van Rianne van der Linde-Preesman. Zij kreeg later gezelschap van de 'pre-B cel student' en huidige voortzetter van het onderzoek, Nico Bos, die op zijn beurt weer kon steunen op Sinka Benne. Rianne en Nico, ik heb jullie inzet en enthousiasme zeer gewaardeerd en stel het bijzonder op prijs dat jullie me wederom willen bijstaan tijdens de promotie.
Ook de huidige en vroegere deelnemers aan de werkbesprekingen immunologie hebben ieder zo hun bijdrage geleverd; de oud-collega's André Bianchi, Guus Koch en Herman Bril wil ik hier met name noemen, ook vanwege de goede contacten die buiten de directe werksfeer lagen.
Adri van Oudenaren, begiftigd met een goede 'immunologische memory' wil ik bedanken voor de reeks lessen praktische immunologie die ik van hem mocht krijgen in mijn eerste tijd in Rotterdam. Zijn kunde, geduld en rustige manier van doen maakten mij snel vertrouwd met de toe te passen technieken.
Ann Tio-Gillen wil ik bedanken voor haar waardevolle adviezen met betrekking tot de Engelse taal.
Voorts wil ik de mensen van de 8e verdieping die op het gebied van de 'humane immunologie' werkzaam zijn speciaal bedanken voor de collegiale houding die zij hebben aangenomen als ik weer eens niet op het lab aanwezig was in verband met het schrijven van dit proefschrift. Jacques van Dongen en Henk Adriaansen, jullie hebben veel opgevangen, geholpen door de onverminderde inzet van de overigen: naast de reeds genoemde Rianne en Sinka, Marieke Comans-Bitter, Ellen Cristen, Ingrid Wolvers-Tettero en op 't laatst ook Anita Bresser, Betsy de Glopper en Annet Wolf. Ook Marjan Reintjes-Versnel en Monique Bouts zijn af en toe bijgesprongen

De muizen en ratten waren in goede handen bij Joop Bos, Joop Brandenburg, Ferry Sievert en Marry en Yvonne Steinvoort.
Een bijzondere plaats neemt Schaap 108 in. Vrijwel alle experimenten, waar schape rode bloedcellen (SRBC) aan te pas kwamen, zijn met het bloed van deze ram, hoewel niet altijd met zijn volle instemming, uitgevoerd. Dr. Philip van der Heiden van het Centraal Diergeneeskundig Instituut (toen nog in Rotterdam) en later Ed Lansbergen van het Centraal Proefdieren Bedrijf van de Erasmus Universiteit, wisten met hun medewerkers onze voorraad SRBC steeds weer op peil te brengen.
Piet Hartwijk heeft een deel van de tekeningen verzorgd en was paraat als er problemen van technische aard waren.
Tar van Os en Joop Fengler verzorgden op kundige wijze de fotografie. Ook de mensen van het Audiovisueel Centrum hebben hun steentje bijgedragen.
Rein Smid verzorgde immer snel en doeltreffend de bestellingen van de benodigde materialen.
Mevrouw Godijn, Jopie Bolman en Lia de Wever zorgden voor schoon glaswerk, steriele instrumenten, de koffie en de thee.
Apart moet nog Cary Meijerink-Clerkx vermeld worden die in een hoog tempo en met grote accuratesse vanuit mijn handgeschreven vellen de publicaties wist te typen en ook het tikwerk van de vele versies van dit proefschrift heeft verzorgd.
Jurjen van Leeuwen en Hella Nubé zorgden op originele wijze voor het ontwerp en de uitvoering van de omslag van dit proefschrift.
Verder wil ik alle medewerkers van de afdeling Celbiologie en Genetica die ik niet met name heb genoemd, van harte bedanken voor hun medewerking en de adviezen die zij bij bepaalde problemen hebben verleend.
Tenslotte wil ik het 'thuisfront' dank zeggen voor het begrip en de ruimte die mij gegeven werd om me naast de vele uren op het lab ook nog thuis bezig te houden met het werken aan dit proefschrift. Bovendien had ik aan Titia een goede hulp bij de proeven met de 'antigeen-vrije' muizen in Notre Dame. Titia, de experimenten beschreven in Hoofdstuk XI hadden zonder jouw hulp niet in die paar weken zo uitgebreid kunnen worden uitgevoerd. De vakantie die erop volgde, was onvergetelijk.



Schaap 108

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 30 mei 1951 te Utrecht geboren. Na het behalen van het diploma Gymnasium β aan de Scholengemeenschap Christelijk Lyceum-HAVO te Gouda in 1970 werd in hetzelfde jaar de studie Biologie aangevangen aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen B1 werd afgelegd in december 1973. De doctoraalstudie omvatte de hoofdrichting Scheikundige Dierfysiologie (onderzoek naar de rol van proteine kinases bij de endocriene regulatie van de lipidstofwisseling in de treksprinkhaan Locusta migratoria onder begeleiding van Prof.Dr. A.M.Th. Beenakkers en Dr. W.J.A. van Marrewijk) en de bijvakken Celbiologie (onderzoek naar het werkingsmechanisme van het melanoforen stimulerend hormoon in de Afrikaanse klauwkikker Xenopus laevis onder begeleiding van Dr. F.C.G. van de Veerdonk) en Biologische Toxicologie (onderzoek naar de cytotoxische en immunosuppressieve werking van organische tinverbindingen in de rat, muis, cavia en mens onder begeleiding van Prof.Dr. H. van Genderen, Dr. W. Seinen en Dr. J.G. Vos) met als nevenrichting de cursus Vakdidaktiek van de Biologie (onder begeleiding van Drs. J. Buddingh, Drs. H. Samwel en P. Timmermans) waarmee de onderwijsbevoegdheid werd behaald. In september 1979 werd het doctoraalexamen cum laude afgelegd. In de periode maart-juni 1979 was de promovendus als vervangend docent biologie werkzaam op het Alberdingk Thijm College te Hilversum. Oktober en november 1979 deed hij literatuuronderzoek naar het energiemetabolisme bij insekten op het Laboratorium voor Scheikundige Dierfysiologie bij Prof.Dr. A.M.Th. Beenakkers. Vanaf 1 december 1979 was hij verbonden aan de vakgroep Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam. Binnen deze vakgroep werd het in dit proefschrift beschreven onderzoek verricht onder leiding van Prof.Dr. R. Benner en Prof.Dr. O. Vos. Sedert 1 februari 1985 is hij hoofd van het immuundiagnostisch laboratorium van de Afdeling Immunologie van het Academisch Ziekenhuis Rotterdam.