B LYMPHOCYTE DIFFERENTIATION IN THE MOUSE

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN
DOCTOR IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT TE ROTTERDAM

OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. B. LEIJNSE
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 1 JUNI 1977 DES NAMIDDAGS
TE 3.00 UUR PRECIES

DOOR

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geboren te Rotterdam in 1949

1977
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Dit proefschrift werd bewerkt binnen de vakgroep Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam. Het onderzoek werd mogelijk gemaakt door financiële steun van het Interuniversitair Instituut voor Radiopathologie en Stralingsbescherming (IRS) te Leiden.

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J. Rozing, N.H.C. Brons and R. Benner.
Cell. Immunol. 29, 37-53 (1977).

Paper II: B Lymphocyte Differentiation in Lethally Irradiated and Reconstituted Mice. III. The Influence of Splenectomy on the Recovery of the B Cell Population.
J. Rozing, N.H.C. Brons and R. Benner.
Cell. Immunol. *in press.*

Paper III: Effects of Splenectomy on the Humoral Immune System. A study in Neonatally and Adult Splenectomized Mice.
J. Rozing, N.H.C. Brons and R. Benner.
Submitted for publication.

Paper IV: B Lymphocyte Differentiation in Lethally Irradiated and Reconstituted Mice. I. The Effect of Strontium-89 Induced Bone Marrow Aplasia on the Recovery of the B Cell Compartment in the Spleen.
J. Rozing, W.A. Buurman and R. Benner.
Cell. Immunol. 24, 79-89 (1976).

Paper V: B Lymphocyte Differentiation in Lethally Irradiated and Reconstituted Mice. IV. A Histological Study Using Immunofluorescent Detection of B Lymphocytes in Sections of Spleen, Lymph Nodes and Peyer's Patches.
J. Rozing, N.H.C. Brons, W. van Ewijk and R. Benner.
Submitted for publication.

GENERAL INTRODUCTION

Introduction

When an antigen enters the body, it can react upon such an invasion with a non-specific and a specific defense mechanism. Phagocytic white blood cells can attack antigens, such as present on the surface of bacteria and viruses, non-specifically by engulfing and destroying these particles. The specific defense against foreign agents depends on the immune system of the individual and can be divided in *cell-mediated* and *humoral* immunity. Cell-mediated immune reactions can be defined as those immunological reactions, which are transferable by cells and not by serum. Cell-mediated immunity includes phenomena like allograft rejection, allogeneic disease, delayed hypersensitivity, and cell-mediated defense against viruses and fungi. The cell type which mediates this type of immune response is the lymphocyte, which is dependent on the thymus for its differentiation: the T lymphocyte. Another function of the T cell is a regulatory influence on humoral immunity. Some aspects of this function of the T lymphocyte will be discussed later.

Humoral immunity is the other possibility for the body in the specific defense against foreign invaders. Humoral immune responses are defined as those immunological responses transferable by serum. Humoral immunity includes manifestations as immediate hypersensitivity, Arthus reactions, and of course antibody-mediated protective immunity. The active substances in the serum, which are responsible for the possibility to transfer humoral immunity, are the antibodies. Antibodies are highly specific proteins with a common structure and they belong to the family of the immunoglobulins. They are transported through the blood stream. Upon interaction with the antigen a sequence of reactions starts. The type of the reaction depends on the class of immunoglobulins to which the antibody-molecule belongs. At present five major structural classes of immunoglobulins can be distinguished: immunoglobulin M (IgM), IgG, IgA, IgD and IgE. IgG is the most important immunoglobulin in quantitative terms. It diffuses more readily than the other immunoglobulin classes in the extra-vascular spaces of the body and this characteristic of IgG makes it suitable to neutralize bacterial endotoxins and to bind microorganisms there. Most IgG molecules are also able to fix another serum protein, i.e. the first complement factor, thereby activating the complement system. IgM molecules are produced early in the immune response. They have a high efficiency to agglutinate, can also fix complement and are very important as an effective first line defense in cases of bacteraemia. IgA is the predominant immunoglobulin in the sero-mucous secretions, where it defends the external surfaces of the body against invading microorganisms. IgE is present in very low concentrations in the serum, it fixes to mast cells and to basophilic granulocytes. Subsequent contact with antigen leads to degranulation of these cells with release of histamine. This process is responsible for the symptomathology of atopic allergy. It has been suggested that a major function

of IgE antibodies is the defence against parasitic infections. The last immunoglobulin is IgD. No particular function for serum IgD has been demonstrated. Besides the distribution over the verious immunoglobulin classes, also called the *isotypes*, there is an enormous diversity in antibodies in the blood, since antibodies are considered to be monospecific. That means that antibodies can react only with one antigen. These antibodies have the same antigen-specificity or *idiotype*. The situation is even more complicated by the presence of genetically determined differences in the immunoglobulin molecule: *the allotypes*. Therefore the number of different antibodies in the blood must be very large. Jerne (1960) once argued that this repertoire could be well over one million. Two main theories exist to explain the genetic basis for such a diversity. Firstly, the germ-line or multi-gene theory, which explains the antibody diversity by assuming that genes coding for antibodies against all possible antigens are already present in the DNA of the zygote and thus in all cells. Antibody diversity would be the result of the activation of different pairs of genes. Secondly, the somatic generation or pauci-gene theory, which assumes the presence of a basic set of immunoglobulin genes, composed during evolution, that in certain lymphoid cells undergo random mutation, thereby generating antibody diversity. Most experts tend to favour the latter.

Antibodies are produced by plasmacells. The first experiments on the cell type that produces antibodies, go back to 1938. Based on the observation of Bing and Plum (1937) that in a large number of cases of aplastic anemia a striking relationship existed between marrow plasmacytosis and hyperglobulinemia, Kolouch (1938) treated rabbits repeatedly with *Streptococcus viridans*. From the observed developing plasmacytosis he concluded that antibody synthesis in his rabbits might depend on the plasmacells appearing in the bone marrow. This suggestions was supported by the work of others (Bjorneboe and Gormsen, 1943) and finally proven by Fagraeus (1948) by means of *in vitro* studies. In these studies she showed that plasmacell-rich spleen fragments produced antibodies and lymphocyte-rich spleen fragments did not. Nowadays, with immunofluorescence microscopy, it is quite simple to recognize plasmacells and plasmablasts as the main source of antibodies and immunoglobulins.

As clear as the relation between plasmacells and antibodies, is today the fact that plasmacells are the progeny of lymphocytes, and especially a certain class of lymphocytes: the B lymphocytes. The major piece of evidence that B lymphocytes are the progenitors of plasmacells, came from experiments in chicken. As will be discussed later, in these animals the development of B cells during ontogeny is dependent on a certain organ: the Bursa of Fabricius. Removal of the Bursa just before hatching results in most cases in a complete failure of such treated animals to produce serum antibodies upon administration of antigen. Although comparable experiments in mammals cannot be performed, since no Bursa of Fabricius or an organ with an equivalent function is present, it is evident from other types of experiments that also in mammals plasmacells are derived from B lymphocytes.

Characteristics of B lymphocytes

DEFINITION OF B LYMPHOCYTES

The most general, but also a negative definition of a B lymphocyte is: thymus-independent lymphocyte, i.e. a lymphocyte that is not dependent on the thymus for its differentiation. However, using this definition, the question arises on which organ the B cell does depend for its differentiation. It would be preferable to mention this organ in the definition of the B lymphocyte. In chicken this is no problem. As mentioned above B lymphocytes in chicken are during a certain stage of their development in ontogeny dependent for their differentiation on the Bursa of Fabricius. Therefore B lymphocytes in chicken can be defined as Bursa-dependent lymphocytes, abbreviated as B lymphocytes or B cells. Mammals do not possess a Bursa of Fabricius. In analogy to the avian model a search started for an organ with a similar function for B lymphocyte differentiation in mammals. Consequently B lymphocytes in mammals have been defined as Bursa-equivalent-derived lymphocytes, also abbreviated as B lymphocytes or B cells. Although no particular organ in mammals seems to have a true Bursa-equivalent function as will be discussed in a later section, and therefore this definition has lost its value, the abbreviation B lymphocyte introduced by this definition is still maintained.

The term B lymphocyte has also been used to indicate B cells as "bone-marrow-derived" lymphocytes. This definition, although probably correct as shown in a later part of this thesis, may be confusing since it is not obvious whether this characterization means that B cells are dependent on the bone marrow for their differentiation, or that the bone marrow is considered as the ultimate source of the cells capable to differentiate into antibody-producing cells. If the latter description is true it must be kept in mind that in adult rodents and also in man the bone marrow is the major source of hemopoietic stem cells, which have the ability to differentiate in B as well as T lymphocytes. In this sense, T lymphocytes are also "bone-marrow-derived" cells.

Finally B cells can also be defined as the progenitors of antibody-forming cells. This definition is used especially in experimental approaches in which B lymphocytes or B lymphocyte sub-populations are determined and quantified using their ability to differentiate into the end-cells of the B cell line, the antibody-producing cells.

TISSUE DISTRIBUTION, MIGRATION, AND RECIRCULATION

Comparison of the number of B cells determined with a surface marker shows a different but rather constant distribution of these cells over the various lymphoid organs (Table 1). Histological investigation of the peripheral lymphoid organs shows that B lymphocytes are not randomly distributed throughout the stroma, but localize in distinct B cell compartments. Studying neonatally thymectomized rats, Waksman

TABLE I

Quantitative Distribution of Surface-Immunoglobulin Positive B Cells in Various Organs of (C57BL x CBA)F1 Mice.

ORGAN	% B CELLS[a]
Spleen	45.4 ± 3.0[b]
Bone marrow[c]	10.3 ± 1.6
Lymph nodes[d]	23.3 ± 1.5
Peyer's patches	48.4 ± 4.9

[a] Percentage of B cells is expressed as percentage of all nucleated cells
[b] Average ± 1 S.E.M. Five mice were tested individually
[c] Bone marrow was obtained from the femoral shafts
[d] Inguinal, brachial, axillary and mesenteric lymph nodes were used

and colleagues (1962) found a selective lymphocyte depletion in the periarteriolar lymphatic sheath (PALS) of the splenic white pulp, in the mid- and deep cortex (paracortex) of lymph nodes and in interfollicular areas of gastro-intestinal lymphoid tissues. However, no effect of neonatal thymectomy was noted in the follicles and peripheral regions of the splenic white pulp, in the lymph follicles and medulla of lymph nodes and in the follicles of gastrointestinal lymphoid organs. Similar observations were described by Parrott et al. (1966) in neonatally thymectomized mice and De Sousa et al. (1969) in congenital athymic nude mice. The areas which are unaffected by T cell depletion are called "thymus-independent" and are considered to be specific B cell compartments. Indeed, B lymphocytes labeled with a radioisotope and injected intravenously appear to localize in "thymus-independent" areas (Sprent, 1973; Gutman and Weissman, 1973; Van Ewijk and Van der Kwast, 1977). Detection of B lymphocytes in frozen sections of lymphoid tissue by means of specific fluorescent anti-sera against B cell markers (Fig. 1) have confirmed the presence of well defined B lymphocyte compartments in peripheral lymphoid organs (Gutman and Weissman, 1972; Weissman et al., 1976; Van Ewijk et al., 1977; Rozing et al. 1977c).

These compartments are in a dynamic state. Cells are continually migrating from and towards other compartments and organs. Lymphocytes from the bone marrow, probably newly formed B lymphocytes, migrate continuously through the blood stream predominantly to the spleen but also to lymph nodes (Brahim and Osmond, 1970; 1973). An identical migration pattern was noted by Sprent (1974) after intravenous injection of radiolabeled thoracic duct lymphocytes of nude mice, probably more mature B cells, into the same strain of mice. Obviously, B lymphocytes of various sources and most likely of different maturation stages follow the same migration pathway between the various organs.

Such a relationship between various B cell subpopulations is even more distinct after comparison of the migration pathway of B lymphocytes from various sources within various lymphoid organs. Van Ewijk and Van der Kwast (1977) used spleen

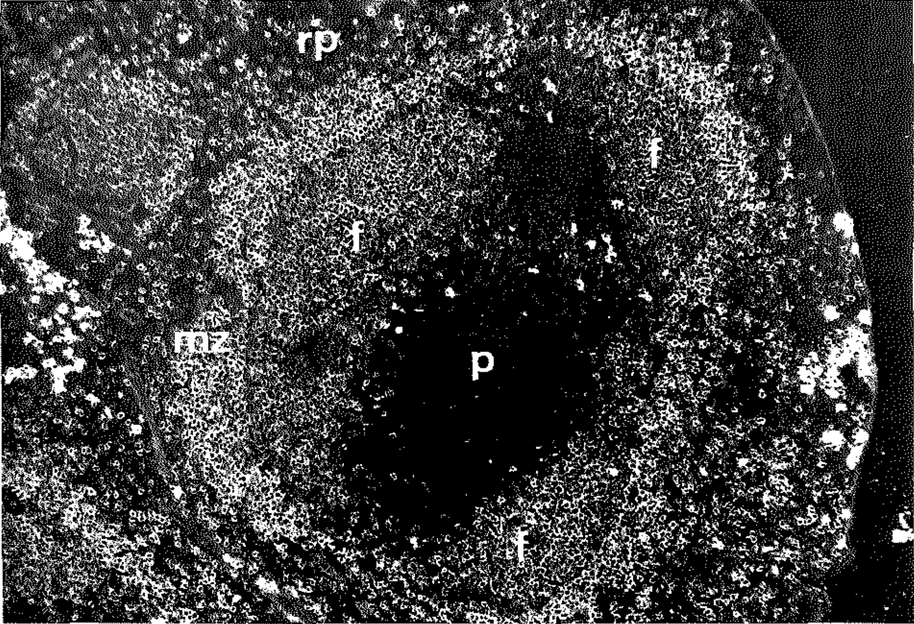

Fig. 1. Frozen section of the splenic white pulp of a normal mouse. The section was incubated with RAM-Ig followed by TRITC-GAR-Ig. Primary follicles (f) and marginal zone (mz) cells are positively stained, whereas the PALS (p) is negative. Strongly fluorescent cells in the red pulp (rp) are plasmacells. (x 120).

cells of T cell depleted mice as a source of B cells. After *in vitro* labeling these B cells were injected intravenously into B cell depleted mice and their migration pattern within spleen and lymph nodes was followed with autoradiography. B lymphocytes entered the white pulp of the spleen mainly via the marginal sinus. Similar observations were made by Goldschneider and McGregor (1968), Ford (1969), and Nieuwenhuis and Ford (1976). However, the most striking point in the data of Van Ewijk and Van der Kwast (1977) is that on their way to the specific B cell areas B lymphocytes migrate through the peripheral and central PALS, which is a "thymus-dependent" area. In their migration studies of newly formed bone marrow lymphocytes, which are probably B lymphocytes, Brahim and Osmond (1970, 1973) noticed that these cells "were non-specifically distributed through B and T cell areas in peripheral lymphoid organs". These authors concluded that the newly formed cells probably lacked a specific membrane determinant involved in the homing of B lymphocytes. Whatever the reason may be, these newly formed B cells also seemed to migrate through thymus-dependent areas. Friedberg and Weissman (1974) observed in the spleen of neonatal mice also a localization of B lymphocytes in the central part of the PALS, whereas the first B cells after lethal irradiation

and fetal liver reconstitution also appear in this region (Rozing et al., 1977c). The results of all these studies can be generalized by arguing that the B cell populations used or determined were generally rather immature, since also the majority of B lymphocytes in the spleen is renewed every 4 days, probably by newly formed immigrants from the bone marrow (S. Strober, personal communication). However, Sprent (1974), studying the migration of labeled thoracic duct lymphocytes from T cell depleted mice after injection of these cells into normal mice, found that after 4 hr a few cells were still located close to the central arteriole in the spleen. The B cells he used are supposed to be more mature B lymphocytes as will be discussed later in this section. Recently Nieuwenhuis and Ford (1976) investigated the migration of B lymphocytes in the rat spleen and lymph nodes. As a B cell source they used thoracic duct lymphocytes of thymectomized, irradiated rats and thoracic duct lymphocyte suspensions of normal rats enriched for B cells. After *in vitro* labeling these cells were injected intravenously into syngeneic recipient rats. Upon entering the white pulp of the spleen B lymphocytes remained at first in the PALS before moving to their specific follicular compartment. Although differences in transit time can occur, it thus seems that also more mature B lymphocytes traverse thymus-dependent areas on their way to specific B cell compartments. As in the spleen, B cells in lymph nodes were found to migrate across T cell areas (Nieuwenhuis and Ford, 1976; Van Ewijk and Van der Kwast, 1977) independent of their stage of maturation and their origin.

B lymphocytes thus seem to follow a specific B cell migration pathway both between and within lymphoid organs. Presumably surface properties of the B lymphocytes play an important role in determining this pattern of migration. Treatment of lymphocytes with enzymes such as neuraminidase or trypsin indeed causes a profound alteration in the homing properties of the cells (Woodruff and Gesner, 1968; 1969; Berney and Gesner, 1970).

Besides migration also recirculation through the lymphoid system occurs. The recirculating pool of lymphocytes has been defined to consist of "those cells that shuttle between blood and lymph" (Greaves et al., 1973). In order to investigate whether a certain cell population can recirculate such cells can be collected, radiolabeled and subsequently injected intravenously into syngeneic recipients. When labeled cells appear in the thoracic duct lymph, these cells are recirculating. Since the thoracic duct is an essential part of the recirculation pathway of lymphocytes most investigators have focussed their attention on cells present in thoracic duct lymph. Continuous drainage of the thoracic duct is thought to remove the recirculating cell pool and depletion of specific lymphoid areas would reflect the compartments through which recirculation occurs (Gowans and McGregor, 1965). The fact that thymus-independent compartments in spleen, lymph nodes and Peyer's patches are hardly depleted by prolonged thoracic duct drainage in rats (Gowans and McGregor, 1965) suggests that most B cells do not recirculate. However, B lymphocytes are present in thoracic duct lymph (Rabellino et al., 1971; Sprent and Basten, 1973; Sprent, 1974) and the question is whether these cells are recirculating or

just transiently present in the recirculating pool. Various studies (Howard, 1972; Sprent and Miller, 1972; Sprent and Basten, 1973; Sprent, 1974) have now indicated that B lymphocytes do recirculate between blood and lymph, only the tempo of this recirculation is much slower than that of T lymphocytes. Howard et al. (1972) showed furthermore that B cell recirculation occurs through the follicular compartments of the peripheral lymphoid organs.

The question what type of B lymphocyte is recirculating through the thoracic duct can be answered by comparing the ability of thoracic duct B lymphocytes of primed and non-primed rats to recirculate after transfer into secondary recipients. Strober (1975) found that only the primed thoracic duct B cells were able to recirculate. In view of these data B lymphocytes present in the thoracic duct lymph probably represent recirculating antigen-experienced (memory) B cells.

The exact functions of the various B cell activities described in this section, i.e. migration and recirculation, are largely unknown. They probably reflect an effective way to ensure an optimal chance for the various lymphocytes to interact with antigen and with each other.

LIFESPAN

The lifespan of a cell is "the interval between two mitotic divisions or between one division and cell death" (Greaves et al., 1973). The incorporation of ^{3}H-thymidine in cells can be used as a tool to investigate the lifespan of cells. Following the accumulation of labeled small lymphocytes in the blood of rats that were continuously treated with ^{3}H-thymidine over a prolonged period of time, Everett et al. (1964) demonstrated that the number of labeled cells increased rapidly during the first 10 days, followed by a more gradual rise after that time. After 270 days about 8% of the small lymphocytes were still unlabeled. These results indicate that two populations of lymphocytes exist according to lifespan: a short-lived and a long-lived population.

At first long-lived lymphocytes have been associated with T lymphocytes and short-lived lymphocytes with B lymphocytes (Davies, 1969). However, numerous studies (Howard, 1972; Sprent and Miller, 1972; Sprent, 1974) indicate that B cells can also be long-lived. Again the question arises: which B lymphocytes are short-lived and which are long-lived? Studies of Brahim and Osmond (1970; 1973) on the proliferation of bone marrow lymphocytes, which are probably B lymphocytes (discussed in a later section), revealed that the majority of the newly formed bone marrow lymphocytes were short-lived. Taken together with the results of Strober (1975) who showed that non-primed cells are short-lived, the population of short-lived B lymphocytes probably reflects the population of newly-formed, antigen-inexperienced (virgin) B lymphocytes. Investigating the lifespan of thoracic duct B cells, which are supposed to be memory B lymphocytes, Sprent and Basten (1973) found an average lifespan for such cells of 5-7 weeks. Although this lifespan seems to be shorter than the average lifespan of T lymphocytes (Sprent, 1974), thoracic

duct B lymphocytes are certainly long-lived. Since Strober (1975), using functional tests, demonstrated that primed cells are long-lived, the population of long-lived B lymphocytes probably reflects the population of antigen-experienced (memory) B lymphocytes.

SURFACE ANTIGENS AND RECEPTORS

The most general marker on the surface of B lymphocytes is *surface immunoglobulin* (sIg). The presence of immunoglobulins on B cells has been shown by direct visualization, using the binding of fluorescent anti-immunoglobulin reagents (Raff, 1970), and by immunochemical studies (Vitetta et al., 1971; Marchalonis et al., 1972). Although the presence of sIg on T lymphocytes has been reported (Marchalonis et al., 1972; Nossal et al., 1972), this is not generally accepted. In general T cells are therefore considered to be surface-immunoglobulin negative. The immunoglobulins bound to the surface of B lymphocytes are synthesized by the cell itself and not passively acquired from the serum-immunoglobulins (Sell, 1970; Pernis et al., 1970; Grey et al., 1971). In analogy to the serum-immunoglobulins several classes of surface immunoglobulins can be determined on B lymphocytes. Approximately 70% of sIg-positive lymphocytes in the spleen of adult mice is carrying IgM on their surface (Abney et al., 1976; Parkhouse et al., 1976), whereas a similar percentage of sIg-positive lymphocytes is IgD-positive (Melcher et al., 1974; Abney et al., 1976; Vitetta and Uhr, 1976). This indicates that a substantial proportion of these cells is IgM as well as IgD-positive. Indeed, double-staining experiments have shown that about 30-40% of sIg-positive lymphocytes in the spleen is both IgM and IgD-positive (Abney et al., 1976; Parkhouse et al., 1976; Vitetta and Uhr, 1976). In these experiments similar percentages of splenic lymphocytes were found to be positive only for IgM or IgD. Estimations about the number of IgG and IgA bearing lymphocytes in the spleen are controversial (Abney et al., 1976; Okumura et al., 1976; Zan-Bar et al., 1977a; 1977b). This may depend on differences in assay procedures or specificity of the anti-sera used.

There seem to be marked differences between virgin and memory B lymphocytes in their expression of the classes of surface immunoglobulins. Whereas virgin B cells are thought to carry IgM or IgD, or both (Zan-Bar et al., 1977b; Coffman and Cohn, 1977) on their surface, the majority of memory B cells has surface IgG or IgA (Okumura et al., 1976; Mason, 1976; Zan-Bar et al., 1977a; Coffman and Cohn, 1977). It is not known whether memory cells have IgD or not. IgA bearing B cells seem primarily to be associated with the gastro-intestinal region, since such cells are predominantly found in Peyer's patches and gut-draining lymph nodes (Craig and Cebra, 1971; Guy-Grand et al., 1974). After transportation through the thoracic duct and the blood stream these cells home to the gut and transform into IgA-producing plasmacells (Guy-Grand et al., 1974; Williams and Gowans, 1975).

There is now substantial evidence that the immunoglobulin molecules on the surface of B lymphocytes are the receptors for antigen (Raff and De Petris, 1973;

Warner, 1974). The surface immunoglobulin on a single B cell is homogeneous with respect to the specificity for antigen (Raff et al., 1973; Julius et al., 1976). Once stimulated by an antigen the progeny of a B lymphocyte, the antibody forming cells, secrete immunoglobulins with a specificity for the antigen identical to that of the surface immunoglobulin of the B cell (Wigzell 1974; Julius and Herzenberg, 1974). Thus antibody diversity as reflected in the serum-immunoglobulins is already present in the B cell population and the generation of this diversity must be accompanied by the generation of large numbers of B lymphocytes.

A proportion of B lymphocytes carries besides surface immunoglobulins other cell surface receptors. A predominant one is the receptor which is capable to react with antigen-antibody-complement complexes: the *complement receptor* or *C_3-receptor* (Bianco et al., 1970). This receptor is also found on phagocytic cells (Parish, 1975) but C_3-receptor positive lymphocytes can be distinguished from these cells by the fact that antigen-antibody-complement binding to phagocytic cells requires divalent cations, while binding to lymphocytes does not (Greaves et al., 1973). The complement receptor is not identical to surface immunoglobulin (Nussenzweig, 1971) nor is it associated with sIg (Parish, 1975). Although the exact function of the complement receptor on B lymphocytes is still unknown a number of suggestions for possible roles in the immune response has been given. For example, it has been suggested that binding of antigen-antibody-complement complexes to the C_3-receptor on B lymphocytes may provide a necessary "second signal" for T cell-independent B cell triggering (Dukor and Hartmann, 1973). The C_3-receptor may also be involved in the presentation of thymus-dependent antigens to B lymphocytes (Feldmann and Pepys, 1974). Evidence against both possibilities has also been presented (Parish, 1975; Pryjama and Humphrey, 1975; Waldmann and Lackmann, 1975). Parish (1975) proposed that complement receptors on B lymphocytes "provide an additional binding site which stabilizes the union between the antigen-specific receptor (sIg) and antigen". As will be discussed in a later section complement receptors on B lymphocytes are possibly also involved in the generation of B memory cells (Klaus and Humphrey, 1977) and antigen-dependent B cell proliferation. Finally it appears from the work of Bloch-Shtacher et al. (1968) that attachment of antigen-antibody-complement complexes to the C_3-receptor of B lymphocytes induces DNA synthesis and proliferation in these cells.

Another surface marker found on B lymphocytes is the receptor for antigen-antibody complexes: the *Fc-receptor* (Uhr and Phillips, 1966). It was shown by Basten et al. (1972) and Paraskevas et al. (1972) that binding of these complexes to lymphocytes could occur in the absence of complement and depends on the presence of the intact Fc part of the immunoglobulin molecule. Fc-receptors are also present on phagocytic cells and on a proportion of T lymphocytes (Parish, 1975). Not all sIg-positive B cells have Fc receptors. Parish (1975) showed that in the spleen of 6-8 week old CBA mice approximately 75% of the immunoglobulin positive B lymphocytes also carried the Fc receptor. Recent studies reported by several groups (Abbas and Unanue, 1975; Basten et al., 1976; Krammer and Pernis,

1976) revealed that Fc receptors are closely associated to surface immunoglobulin, especially of the IgM-class (Forni and Pernis, 1975). The role of Fc receptors on B lymphocytes is still unknown, but a number of suggestions has been made.

(1) Ramasamy et al. (1974) proposed that they function as receptors for endogenously produced membrane immunoglobulin. This suggestion seems to be in agreement with the above mentioned close association between Fc receptors and surface immunoglobulin. However, in that case, one has to assume that surface immunoglobulin is interacting with a site of the Fc receptor which is different from the site which interacts with exogenous immunoglobulin. In this view it may be of importance to note that surface IgM, which is the predominant sIg class on lymphocytes, is 7-8S, while serum IgM is 19S (Melchers et al., 1975). Although primarily exogenous IgG and nog IgM is bound to the Fc receptor (Basten et al., 1972) there may well be competition between endogenously produced 7-8S IgM and exogenous IgG for the same site of the Fc receptor.

(2) Another function for Fc receptors on lymphocytes could be the transport of antigen to and the localization in certain structures of lymphoid organs. This role of Fc receptors is suggested by experiments of Miller et al. (1971) who showed that B cells through their Fc receptor could carry antigen into the spleen. However, in mice depleted of complement, aggregated Ig can no longer be localized in the spleen (Papamichail et al., 1975), suggesting a primary role for the complement receptor and not for the Fc receptor in the process of antigen localization.

(3) Antibodies are known to influence the immune response (Uhr and Moller, 1968). Therefore Fc receptors may function in the regulation of the immune response, since such influences seem to require an intact Fc part of the immunoglobulin molecule.

(4) Parish (1975) suggested a role for the Fc receptor identical to the role of the C_3-receptor, namely that Fc-receptors stabilize the binding between antigen and surface immunoglobulin.

Antigens determined by the Ir (immune response) gene region of the major histocompatibility gene complex of the mouse, the H-2 complex, appear also on the surface of the majority of B lymphocytes (Abbas et al., 1976). These alloantigens are called *Ia-antigens* (Ir-region associated antigens). Besides on B cells Ia antigens have been demonstrated on macrophages (Unanue et al., 1974) and on T cells (Frelinger et al., 1974; Fathmann and Sachs, 1975). Ia antigens on B lymphocytes are not associated with surface immunoglobulins (Abbas et al., 1976), but seem definitely to be associated with Fc receptors (reviewed by Dickler, 1976). Ia antigens on B cells are distributed non-randomly in microclusters (Abbas et al., 1976). These antigens may play a role in the regulation of the immune response (Sachs and Dicker, 1975), or in cell-cell interactions responsible for the generation of immune responses (Katz et al., 1975).

Recently, a new surface component on B lymphocytes has been described by Huber et al. (1977). This surface component, named *Lyb-3,* seems to be present on 50% of all B cells (Cantor and Boyse, 1977). The function of the Lyb-3 surface

component may be to act as a receptor for a T cell signal which, together with antigen triggers B cells (Cantor and Boyse, 1977).

Finally, the *mouse-specific B lymphocyte antigen* (MBLA), is present on the surface of bone marrow and peripheral B lymphocytes, antibody-secreting cells, myeloma cells and some leukaemic cells, but not on T lymphocytes (Raff et al., 1971). The function of MBLA on B lymphocytes is unknown.

The above mentioned cell surface receptors are probably not bound to a single place in the membrane. Most if not all surface proteins can move freely in the plane of the membrane at physiological temperatures (Raff and De Petris, 1973). Antigen may induce redistribution of specific receptors (Taylor et al., 1971) and perhaps this receptor distribution may be important for lymphocyte triggering.

TABLE II

Surface Antigens and Receptors on B Lymphocytes

Determinant	Cell type on which the receptor is also present	Supposed function(s)	Association with other receptors
Surface immunoglobulin (sIg)	(T cells)	antigen receptor	Fc (IgM)
Complement receptor	phagocytic cells	1. additional binding site 2. antigen presentation 3. "second signal" receptor	–
Fc receptor	phagocytic cells T cells	1. receptor for endogenous Ig 2. regulation of the immune response 3. antigen transport 4. additional binding site	IgM Ia
Ia antigen	macrophages T cells	1. regulation of the immune response 2. cell-cell interaction	Fc
Mouse-specific B lymphocyte antigen (MBLA)	plasma cells myeloma cells leukaemic cells	unknown	unknown
Lyb-3 antigen		T cell signal receptor	unknown

B Lymphocyte differentiation in rodents

ANTIGEN-INDEPENDENT B CELL DIFFERENTIATION

In order to study cellular differentiation one has to define the differentiation characteristics of the cell population that is investigated. Differentiation characteristics of cells are those features that are specific for these differentiated cells. The characteristics can be used to distinguish between various cell types in the body, or between various classes of the same cell type (cf. T and B lymphocytes), or even between cells in various stages of their differentiation within the same subclass of a certain cell type (i.e. in the present study the B lymphocyte).

A number of features has been used to define the various subpopulations of B lymphocytes in the various stages of the differentiation process: (a) pure morphological criteria (Rosse and Yoffey, 1967; Rosse, 1976), (b) the presence or density of surface receptors (Osmond and Nossal, 1974a; 1974b; Ryser and Vassalli, 1974; Osmond, 1975; Strober, 1975), (c) the reactivity against certain stimulatory agents (Ryser and Vassalli, 1974, Melchers et al., 1975; Gronowicz and Coutinho, 1975), (d) biophysical properties, such as surface charge (Osmond, 1975; Melchers et al., 1975), (e) the differentiation pattern upon antigenic stimulation (Lafleur et al., 1973; Miller and Phillips, 1975; Miller et al., 1975) or (f) a combination of these differentiation "markers".

It is now generally accepted that the pluripotent hemopoietic stem cell (HSC) detected by the spleen-colony assay of Till and McCulloch (1961) represents the stem cell for both the myeloid and the lymphoid system. Evidence for this latter function of the pluripotent HSC came from studies of Wu et al. (1968) and of Nowell et al. (1970) who showed that besides in cells of the myeloid series, unique radiation induced chromosome markers were present in cells of the thymus and of lymph nodes of mice grafted with marked stem cells (Wu et al., 1968). The same marker was also present in cells stimulated in a mixed leukocyte reaction (Nowell et al., 1970). The latter results suggest that at least T cells are among the progeny of hemopoietic stem cells. Experiments performed by Edwards and colleagues (1970) strongly suggest that also B lymphocytes are derived from the pluripotent hemopoietic stem cell. Edwards and coworkers (1970) repopulated anaemic W/W^v mice with the progeny of one single hemopoietic stem cell, bearing a unique, radiation induced chromosome marker. Upon immunization with sheep red blood cells (SRBC) a spleen cell suspension of these mice was prepared at the peak of the response. Subsequently rosettes were formed and purified. Analysis of mitoses in these rosette-forming cells showed that a large number contained the specific chromosome marker. Since Edwards et al. (1970) worked under conditions in which T cell rosettes are not stable and therefore are mostly excluded, these data suggest that B cells are also among the progeny of pluripotent hemopoietic stem cells. The restriction must be made that, although unlikely, in these latter experiments the

chromosome marker bearing rosette forming cells could also be rosette forming monocytes. Definite evidence for the relationship between hemopoietic stem cells and B and T lymphocytes can be expected in the near future when classical experimental approaches will be combined with elegant cell sorting techniques as made possible with the fluorescence-activated cell sorter developed by Herzenberg (Herzenberg et al., 1976).

Indirect evidence that B lymphocytes are derived from the same hemopoietic stem cells as the other myeloid cell types came from the work of Rosse and Yoffey (Rosse and Yoffey, 1967; Rosse, 1976). On morphological grounds they presented evidence that the precursor of bone marrow small lymphocytes, the majority of which are B lymphocytes, is found in a cell population with the characteristics of a lymphoid cell: the transitional cell. The size of transitional cells in the bone marrow ranges from 8 to 14 μm and they are not identical to medium and large lymphocytes or to lymphocytes transformed by mitogens (reviewed by Rosse, 1976). Based on a different content in RNA the transitional cell population can be divided into "basophilic" and "pale" transitional cells (Rosse, 1976). The pale cells are probably derived from basophilic cells. Although it seems to be at least debatable to classify cells according to the basophilia of their cytoplasm there is a number of studies that indicate that basophilic and pale transitional cells differ in their proliferative behaviour (Rosse, 1973; Miller and Osmond, 1973) and in their response to various hemopoietic stimuli (Rosse, 1973; 1976). The ability to differentiate into cells of the erythroid, granuloid and monocytic series seems to be held by the basophilic transitional cell populations (Rosse, 1976). Transitional cells are proliferating (Yoffey et al., 1965; Yoshida and Osmond, 1971; Miller and Osmond, 1973) with the restriction that the basophilic cells have a higher capacity to proliferate than the pale cells (Rosse, 1970; 1973; Miller and Osmond, 1973). It has been calculated by Rosse (1976) that the rate of proliferation in the transitional cell population exceeds the proliferation which is required to replace all small lymphocytes. This probably reflects the cell renewal necessary for the self-maintenance of the transitional cell compartment and for the replacement of other cell types, such as erythroblasts, granulocytes, and monocytes.

Although transitional cells vary considerably in size (8-14μ) no attempt has been made to classify them according to their size since Rosse (1972) using microcinematography *in vitro* clearly showed that changes in size are related to the cell cycle and that daughter cells that were smaller at first attained later the size of the parent cell before division. Nevertheless a clear relationship between proliferative capacity and cell size of transitional cells has been found (Miller and Osmond, 1973).

There seems to be a good morphological resemblance between the pale transitional cell and the candidate stem cell as described by Van Bekkum and coworkers (Van Bekkum et al., 1971; Dicke et al., 1973). Murphy et al. (1971) however, produced bone marrow fractions enriched for stem cells in which stem cell function was assigned to basophilic transitional cells.

In view of these data it seems plausible that the precursors of B lymphocytes can be found among the population of transitional cells. The question remains whether

certain transitional cells are already committed to one differentiation line or that all transitional cells still have the capacity to differentiate into all cell lines. Although transitional cells in mice are sIg negative, pale transitional cells in the bone marrow of guinea pigs have been reported to be immunoglobulin positive (Rosse, 1976). This latter result would be in favour of the hypothesis that at least certain pale transitional cells are already committed to a single line of differentiation.

For the early differentiation events of the B cell line in rodents a number of models has been proposed by various groups. The definition of the B lymphocyte subpopulations in these models is closely related to the methodology which is used for their determination. Differences between the various models may therefore be related largely to differences in methodology, as will be discussed later in this section. Before introducing another model for B cell differentiation the most important concepts on B lymphocyte differentiation by various groups will first be summarized.

(1) In their studies about the development of B lymphocytes, which respond to sheep red blood cells in mice, Lafleur and colleagues (Lafleur et al., 1973; Miller and Phillips, 1975; Miller et al., 1975) proposed the following model of the B cell differentiation pathway: Precursor B cells, designated as PB cells, differentiate into transitional B_1 cells, which in turn differentiate into B_2 cells. These stages have been defined by the time necessary for these cells to develop into antibody forming cells: a functional definition. Subsequently velocity sedimentation cell separation has been used to characterize these cells. Both PB and B_1 cells are rather rapidly sedimenting cells and are therefore considered to be large cells, while the sedimentation pattern of B_2 cells suggests that these cells are somewhat smaller. Both PB, B_1 and B_2 cells are destroyed by treatment with anti-immunoglobulin serum, suggesting the presence of surface immunoglobulins on all three subclasses of B lymphocytes. The B_2 lymphocyte is the only cell type in this model, which has the capacity to react immediately upon antigenic stimulation.

(2) Osmond and coworkers (Osmond and Nossal, 1974a; 1974b; Osmond, 1975; 1976) investigated the differentiation of bone marrow lymphocytes in mice. They observed that the immediate precursor of the small immunoglobulin positive B lymphocyte is a small immunoglobulin negative lymphocyte. The transformation can occur both *in vivo* and *in vitro* and does not depend on proliferation. Surface immunoglobulins on B cells in these studies have been demonstrated by autoradiography. The immunoglobulin negative precursor B lymphocyte seems to be highly proliferating, whereas the B cell is not. Other surface markers have been studied as well and from the relative percentages of cells with the various surface receptors present in the bone marrow the following sequence of appearance on the B cell surface has been suggested: MBLA, Fc receptor, kappa-light chain, IgM and complement receptor. At least for Fc receptors, surface immunoglobulins and complement receptors it has been demonstrated that their appearance proceeds rather autonomously. During maturation the electrophoretic mobility of newly formed lymphocytes is reduced significantly, suggesting a weakening of their nega-

tive surface charge. There seems to be no direct relationship between the stage of differentiation of the B cell as determined by the presence of surface receptors such as surface immunoglobulin, Fc receptor or C_3 receptor and the electrophoretic mobility of the cell.

(3) Closely related to the model as proposed by Osmond and colleagues are the findings of Ryser and Vassalli (1974). These authors also studied the differentiation of mouse bone marrow lymphocytes. Their model of B cell differentiation is highly comparable to that of Osmond et al. Small immunoglobulin negative "null" lymphocytes acquire subsequently MBLA, kappa light-chain, IgM and the complement receptor on their surface. The sequence of the appearance of these surface receptors has been determined in this study directly by double labeling techniques. Surface immunoglobulin positive B lymphocytes in the spleen, which are considered to be more mature than immunoglobulin positive B lymphocytes in the bone marrow, differ in two aspects: bone marrow B lymphocytes have little or no complement receptors and can hardly respond *in vitro* to the B cell mitogen *Escherichia coli* lipopolysaccharide (LPS) whereas splenic B lymphocytes do have complement receptors and can be stimulated by LPS.

(4) According to Strober (1975), the most primitive member of the B cell line in rats is a large immunoglobulin negative proliferating bone marrow cell, which develops through an immunoglobulin negative small daughter cell into an immunoglobulin positive small lymphocyte. The functional activity of this latter type of B lymphocyte seems to be rather low as measured by its ability to restore the adoptive primary antibody response. Strober (1975) suggests that these immature, small cells gain surface immunoglobulin and enlarge, thereby transforming into large functionally active cells which are clearly immunoglobulin positive. They start to proliferate and produce again small immunoglobulin positive B lymphocytes. Size determination in these studies was performed using velocity sedimentation. For the maturation sequences involved in the acquisition of functional activity of the B lymphocytes in rats, in particular the environment of the spleen seems to be obligatory.

A similar enlargement of small immunoglobulin positive B cells resulting in large functional active immunoglobulin positive B lymphocytes has been described by Shortman et al. (1976) in the spleen of 7 days old neonatal mice.

(5) Finally the experiments of Melchers and colleagues (1975) on the classification of the various subpopulations of B lymphocytes are very interesting. The criteria used by these investigators to characterize the different subpopulations of B cells were immunoglobulin synthesis, surface representation and turnover, size, electrophoretic mobility, and reactivity against various mitogens. Size determination in these studies was done with velocity sedimentation. B lymphocyte subpopulations were analyzed in different lymphoid organs of adult mice as well as during their development in ontogeny. B cell subpopulations that are simultaneously present in the lymphoid tissues of adult animals seem to be identical to those appearing successively during the embryonic and neonatal period. According to the criteria mentioned above and their sequence of appearance during ontogeny Melchers et al.

(1975) suggest the following classification: The first identifiable cell of the B cell lineage is a large immunoglobulin synthetising cell. The endogenously produced 7-8S IgM is incorporated in the cell membrane but it turns over very rapidly (t½ between 1 and 3 hours). This cell has a high electrophoretic mobility and cannot be stimulated by the mitogens LPS and purified protein derivate of tuberculin (PPD). This cell type is classified by these authors as B lymphocyte type I. This large cell type probably differentiates into a small lymphocyte called B lymphocyte type II. This latter cell type is also producing immunoglobulin which can be incorporated in the membrane, but the turnover time of the produced 7-8S IgM is much slower (t½ between 20 and 28 hours). The electrophoretic mobility of type II cells is low and the cells are more or less susceptible to LPS stimulation. The next cell in the differentiation sequence of the B cell line, the type III B lymphocyte, is a lymphocyte with the same characteristics as the type II B lymphocyte only differing from that cell in its capacity to react upon stimulation with LPS and PPD. In general this cell type represents the normal mature B lymphocyte.

It is obvious that there exist numerous controversies between the B cell differentiation models as proposed by the various groups. However, when the limitations of the results obtained with various techniques are taken into account, the various proposals can be brought into line.

The main controversy between the models as suggested by Lafleur and colleagues (Lafleur et al., 1973; Miller and Phillips, 1975; Miller et al., 1975) on one side and the other groups (Ryser and Vassalli, 1974; Strober, 1975; Osmond, 1975; 1976) on the other side seems to be that according to Lafleur et al. the precursors of B lymphocytes are large immunoglobulin positive (PB) cells whereas both Osmond and Nossal (1974a; 1974b) and Ryser and Vassalli (1974) presented evidence that the immediate precursors of immunoglobulin positive small B lymphocytes are small immunoglobulin negative lymphocytes. However, it must be remembered that the PB cell has been defined functionally (Lafleur et al., 1973), while the transition from immunoglobulin negative into immunoglobulin positive small lymphocyte is based on direct microscopic observations (Osmond and Nossal, 1974a; Ryser and Vassalli, 1974).

Also comparison of the results obtained by various techniques for size determination may have added to the confusion. Cell sizes in some experiments have been determined by velocity sedimentation (Lafleur et al., 1973; Strober, 1975; Melchers et al., 1975; Miller and Phillips, 1975; Miller et al., 1975; Shortman et al., 1976) while in other studies morphological characterization has been used (Osmond and Nossal, 1974a; 1974b; Ryser and Vassalli, 1974). Although the sedimentation rate is determined primarily by cell size, it is also influenced by cell density. Therefore, cells with the same sedimentation velocity can differ in size and cells with different sedimentation velocities can have the same size (Miller and Phillips, 1974; Moon et al., 1972). This effect is clearly demonstrated by thoracic duct and splenic B cells. Thoracic duct B lymphocytes are on the average somewhat smaller than splenic B cells, but since thoracic duct B cells are more dense, their sedimentation charac-

teristics are identical to splenic B cells (Shortman et al., 1976). PB cells, for instance, with the sedimentation characteristics of large cells, according to Miller and Phillips (1975) are classified by the same authors on morphological criteria as small lymphocytes. Furthermore, cells that are actively proliferating may also display changes in cell size during the cycle (Rosse, 1972).

An ambuigity can also be noted in the appreciation of surface immunoglobulin positivity. In some studies (Lafleur et al., 1973; Miller and Phillips, 1975) cells have been investigated using the inhibition of such cells by anti-immunoglobulin serum as a criterium, while in the majority of investigations on B lymphocyte differentiation (Osmond and Nossal, 1974a; 1974b, Ryser and Vassalli, 1974; Strober, 1975; Shortman et al., 1976) B cells have been studied with immunofluorescence or radioautographic techniques to determine antiglobulin binding. Perhaps not all cells positive according to the first definition are also positive when tested by the latter assay. These differences in interpretation may also contribute to an even greater confusion about B cell differentiation.

As mentioned above Melchers et al. (1975) described a cell type in an early stage of the B cell differentiation pathway, which produces 7-8S IgM that is incorporated into the cell membrane. The turnover of this surface-IgM however, is "so high that these lymphocytes in cell labeling experiments probably are surface immunoglobulin negative". Nevertheless these cells may well be inhibited by anti-immunoglobulin serum treatment. Perhaps these cells represent the PB cells of Lafleur et al. (1973). Additional evidence for this view may come from the work of De Luca et al. (1974). These authors showed that certain medium-sized bone marrow lymphocytes could bind antigen if these cells were fixed with glutaraldehyde, while no antigen binding was found without fixation or even in the presence of azide. Contradictory to this hypothesis would be the fact that PB cells or at least cells in the same sedimentation region as PB cells have been shown to be immunoglobulin positive by means of membrane fluorescence (Miller and Phillips, 1975). These results may suggest that there exists a certain heterogeneity for immunoglobulin positivity amongst PB cells.

An attempt has been made to correlate the partly conflicting information presented in the literature. This resulted in the following scheme of antigen-independent B lymphocyte differentiation (Fig. 2). In this scheme no indications of cell size or changes in cell size are given in view of the already mentioned criticisms on the classification of cells in subpopulations according to size. The most primitive member of the B cell line is probably the *basophilic "transitional" cell.* This cell is surface-immunoglobulin negative as defined by direct membrane immunofluorescence procedures. The basophilic "transitional" cell differentiates into a *pale "transitional" cell,* which is also surface immunoglobulin negative. The pale "transitional" cell can probably not be distinguished from the following cell in the differentiation pathway. This cell type can be considered as the first of three cell types, that may be characterized as PB cells according to Lafleur and colleagues (Lafleur et al., 1973; Miller and Phillips, 1975) or B lymphocytes type I according to Melchers et al. (1975). The supposed PB_1 cell is still surface immunoglobulin negative

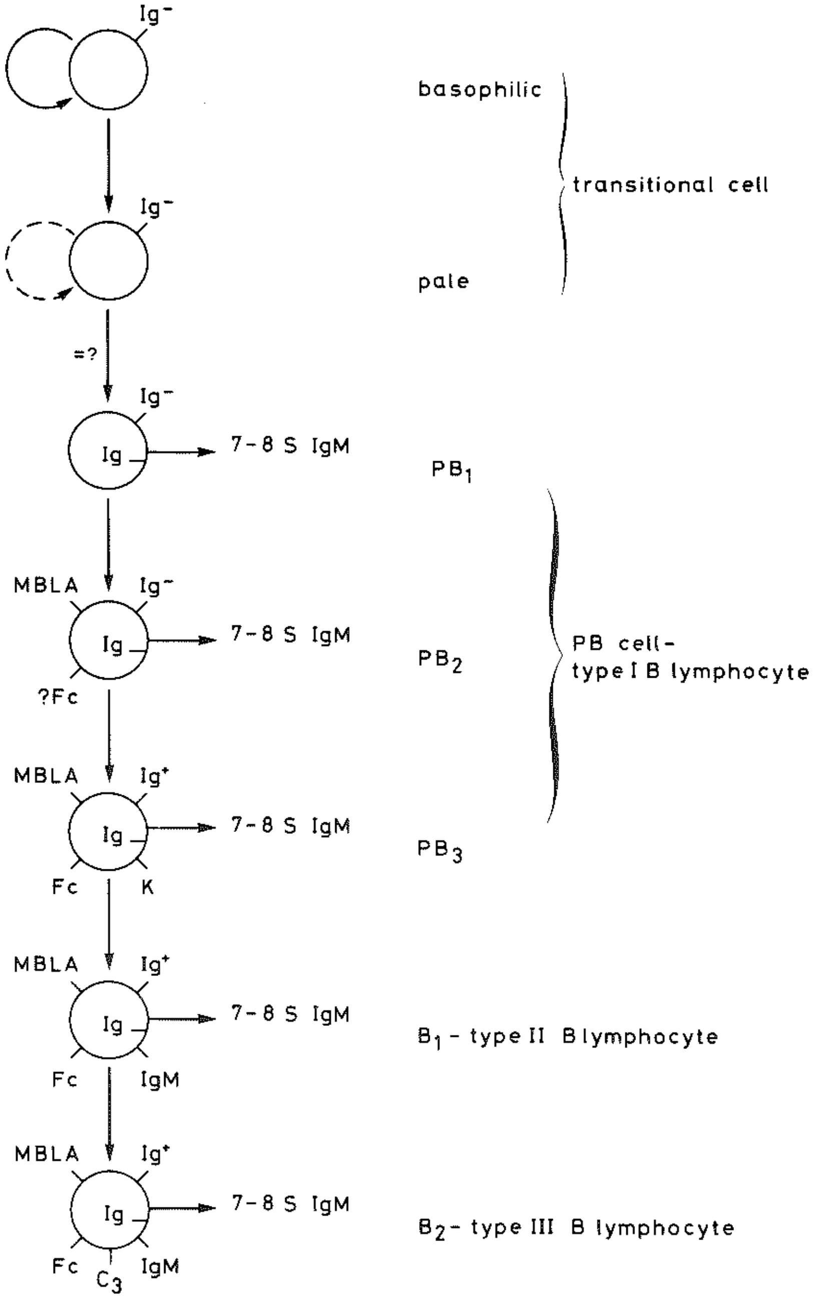

Fig. 2. Scheme of antigen-independent B lymphocyte differentiation.

but produces already 7-8S IgM, which is accessible to the cell surface. The turnover of this surface IgM, however, is so high that the PB_1 cell remains still immunoglobulin-negative in the immunofluorescence test. The only difference between this cell and the *PB_2 cell* would be the appearance of MBLA and probably also of the Fc receptor on the surface of the cell. The first cell on the differentiation pathway of B lymphocytes that can be recognized as immunoglobulin positive defined by direct visualization is the *PB_3 cell.* Although this cell type is still producing 7-8S IgM that is shed off the membrane too rapidly to be determined by cell labeling techniques, single kappa light-chain molecules appear on the surface of these cells. Since Osmond and coworkers (Osmond and Nossal. 1974a; 1974b; Osmond, 1975) used an antiserum with activity a.o. to kappa light-chains to determine immunoglobulin positivity these PB_3 cells have been classified by them presumably as "immunoglobulin positive lymphocytes". Furthermore Fc receptors are present on this cell type.

The PB_3 cell differentiates into a cell type that can be classified as the *B_1 cell* of Lafleur et al. (Lafleur et al., 1973; Miller and Phillips, 1975) or as the *B lymphocyte type II* of Melchers et al. (1975). This cell type produces also 7-8S IgM which is incorporated in the membrane. The slow turnover of the surface IgM allows these cells to be recognized as immunoglobulin positive. The so-called B_1 cell is capable to respond more or less to stimulation with LPS. The transition from the B_1 cell into the last cell type of the antigen-independent B lymphocyte differentiation involves among others the appearance of the complement receptor on the cell surface. This last cell type that seems to be identical to the *B_2 cell* of Lafleur et al. (Lafleur et al., 1973; Miller and Phillips, 1975) or the *B lymphocyte type III* of Melchers et al. (1975) can be stimulated by both LPS and PPD and is supposed to be functionally mature.

The appearance of surface immunoglobulin as well as of Fc and C_3-receptors during differentiation seems to be an autonomous process that, once started, continues (Osmond, 1976). Definite commitment to the B cell lineage probably occurs in an early phase somewhere in the region of the pale "transitional" cell and the PB_1 cell.

Besides the proliferative capacity of the cells in the early stages of B cell differentiation, i.e. the "transitional" cells, such an ability to proliferate may also appear in the cell types classified as B_1 cells (Miller and Phillips, 1975) and B_2 cells (Storber, 1975). However, since immunoglobulin positive lymphocytes are reported to be non-proliferating cells (Osmond and Nossal, 1974a; 1974b) perhaps proliferation is associated with the disappearance of surface immunoglobulins as suggested for B_1 cells by Phillips and Miller (1975). Possibly surface receptors disappear during the S-phase, in which the cells are synthetizing DNA. Indeed Thomas and Phillips (1973) observed an alteration of cell surface antigens during the cell cycle. This would implicate that cells defined as in cycle by pulse-labeling with ^{3}H-thymidine are automatically immunoglobulin negative, whereas the same cell when it is in G_1 or G_2 will be considered as both non-proliferative and immunoglobulin positive. Transition from an immunoglobulin negative lymphocyte into an immunoglobulin

positive lymphocyte as observed by Osmond and Nossal (1974a; 1974b) and Ryser and Vassalli (1974) could then occur on several places of the B cell differentiation pathway.

The first class of surface immunoglobulin appearing on B cells during differentiation is IgM (Cooper et al., 1976), which is followed somewhat later by the immunoglobulin classes IgD, IgG and IgA (Lawton et al., 1972; Vossen, 1975; Cooper et al., 1976). From experiments using anti-IgM treatment *in vivo* in neonatal mice during prolonged periods of time and the resulting depression of IgM as well as IgD, IgG and IgA bearing cells in these animals (Cooper et al., 1976), it can be concluded that the IgM bearing cell is the common progenitor for the cells carrying the other classes of immunoglobulins. The appearance of IgG and IgA reflects presumably antigen-dependent changes (Mason, 1976; Okumura et al., 1976) and will therefore be discussed in another section of this thesis.

Finally it must be kept in mind that B cell differentiation is most likely a continuous process in which one stage gradually goes over into the next stage. Any rigid classification of B lymphocytes in subpopulations has to be considered therefore as artificial.

SITES OF B CELL DIFFERENTIATION

Although B cell differentiation in chicken is somewhat beyond the scope of this section, the organ responsible for the differentiation in this species must be mentioned since it has some implications for the localization of the B cell differentiation in rodents, namely the *Bursa of Fabricius.* The bursa is a lymphoid organ that arises as an evagination of the dorsal wall of the cloaca on the 5th day of incubation. If embryos of chicken are bursectomized at 17 days of incubation, this procedure will prevent in most cases the development of cells of the B cell line resulting in complete agammaglobulinaemia (Cooper et al., 1969; 1972). After that time the bursa seems to lose gradually its important function for B cell differentiation since bursectomy in adult chicken does not affect seriously the humoral immune response (Cooper et al., 1969, 1972). Jankovic (personal communication) found a normal B cell system after hatching in chicken in which on the second day of incubation the part of the body, in which 3 days later the bursa would have developed, had been removed surgically. Lerner et al. (1971) also showed that bursectomy produced by testosterone treatment during early embryogenesis had little effect on IgM-synthesis during adult life. It seems from these data that even after total bursectomy the B cell system can develop in chicken, suggesting a less essential function for the bursa in B cell differentiation as was supposed. Obviously other organs in chicken can compensate for the absence of the Bursa of Fabricius in the process of B cell differentiation.

Nevertheless, the first search for the site where B cell differentiation occurs in mammals has been performed to designate an organ with the same functions and properties as the bursa, namely the *Bursa equivalent.* Although a number of indica-

tions pointed to the lymphoid tissue associated to the gastro-intestinal tract, no formal proof for this hypothesis has been given. On the contrary, Friedberg and Weissman (1974) even presented evidence that the gut-associated lymphoid tissue can not be held responsible for the production of the B lymphocytes present in the entire body.

Even before the separation of lymphocytes into B and T lymphocytes had been established a large scale production of small lymphocytes in the *bone marrow* of guinea pigs had been demonstrated by Osmond and Everett (1964). Similar results have been found in other rodents (Craddock, 1965; Yoffey and Courtice, 1970; Ropke and Everett, 1973; Osmond et al., 1973) and it is estimated that a total production of approximately 10^8 lymphocytes per day occurs in the bone marrow of mice (Osmond, 1976). It became evident from the work of Osmond and Nossal (1974a; 1974b) and Ryser and Vassalli (1974) that this enormous proliferation of small bone marrow lymphocytes represents the continuous production of B lymphocytes. These authors demonstrated that about 50% of bone marrow small lymphocytes is immunoglobulin negative, whereas the other 50% displays surface immunoglobulin in various densities. They were able to show by both *in vitro* cultures and *in vivo* transfer experiments that these cells were all in various maturation stages and that immunoglobulin negative lymphocytes could develop without proliferation into immunoglobulin positive cells.

However, for the acquisition of certain surface receptors, i.e. C_3 receptors, and of complete functional reactivity B lymphocytes seem to need the environment of the spleen (Ryser and Vassalli, 1974; Strober, 1975; 1976a). A continuous migration of newly formed lymphocytes has been demonstrated from the bone marrow into the spleen and also into lymph nodes (Brahim and Osmond, 1970; 1973).

Since hemopoietic stem cells at least in mice also occur in the *spleen* (Metcalf and Moore, 1971) it is most likely that early B cell differentiation can also take place in that organ. The magnitude of the contribution of this splenic B cell differentiation is not known. Direct indications for a role of the spleen in early B cell differentiation came from experiments of Phillips and Miller (1974) and Kincade et al. (1975) in which they treated mice with strontium-89 to eliminate the bone marrow as a place for B cell differentiation. These authors found that B cell differentiation in the strontium-89 treated animals could occur in the spleen. From these experiments it can be concluded that the bone marrow is not indispensable for B cell differentiation in mice.

The notion that B cell differentiation in mice is not assigned to a single organ but probably to all hemopoietic tissues is supported by experiments of Owen et al. (1974). They showed that in cultures of early *fetal liver* tissue of mice immunoglobulin positive lymphocytes developed. In this view also the suggestion of Ozer and Waksman (1970), Davies and Carter (1972) and more recently of Nieuwenhuis and Keuning (1974) about the localization of the B cell differentiation must be mentioned. These authors hypothesize that *germinal centers* are sites at which non-specific amplification of the B cell system can occur. However, since the appearance

of germinal centers is antigen dependent, this aspect of B cell differentiation will be discussed in the section dealing with antigen-dependent B cell differentiation.

FACTORS REGULATING B CELL DIFFERENTIATION

The homeostatic mechanisms involved in the regulation of the process of antigen-independent B cell differentiation are largely unknown. The generation of B lymphocytes in the bone marrow is definitely not dependent on thymic influences since both in neonatally thymectomized and congenital athymic mice the number of B lymphocytes and the turn-over of these cells in the bone marrow are comparable to normal mice (Osmond, 1976; Miller and Osmond, 1976). A splenic influence on B cell differentiation may exist. In neonatally splenectomized mice, adult splenectomized mice and adult splenectomized, irradiated and fetal liver reconstituted mice the number of B lymphocytes in the various organs and especially in the bone marrow is significantly higher than in the mice of the control groups (Rozing et al., 1976; 1977a; 1977b). Perhaps this splenic influence is identical to the regulatory influence of the population size as suggested by Rosse (1976).

A highly controversial observation seems to be the fact that the B cell differentiation in the bone marrow which is supposed to be antigen-independent probably is regulated to some extent by the antigen level. Although normal numbers of B lymphocytes are found in the bone marrow of germfree mice the turn-over of the bone marrow lymphocyte population is prolonged significantly (Osmond and Nossal, 1974a; Osmond, 1975; 1976).

Indications that hormonal influences may be involved in B cell differentiation came from experiments in chicken. Jankovic and Leskowitz (1965), working with bursal tissue in diffusion chambers implanted in bursectomized chicken, found a restoration of antibody production in these animals. These results suggest a hormonal regulatory influence of the bursa on B cell differentiation. However, Dent et al. (1968) presented evidence against this view by demonstrating that the effects as found by Jankovic and Leskowitz (1965) were presumably the result of contaminating bacteria in the bursal grafts.

B lymphocyte differentiation may depend on a microenvironmental induction comparable to the "hemopoietic inductive microenvironment (HIM)" as postulated for the myeloid cell lines (Metcalf and Moore, 1971). Circumstantial evidence for such environmental influences may be derived from experiments of Ritter and Owen who showed that disruption of the follicular structure of bursal tissue in organ cultures completely abolished lymphopoiesis, whereas in carefully cultured bursal tissue lymphocytes develop normally (Greaves et al., 1973).

It is obvious that one of the major questions that is still open in the process of B lymphocyte differentiation concerns the identification of influences regulating this process, especially the ones that are regulating the commitment of cells to the B cell lineage. Further experimental analysis of this problem is clearly needed.

ANTIGEN-DEPENDENT B CELL DIFFERENTIATION

Contact of a virgin B cell with the appropriate antigen can result in two different differentiation pathways: the maturation into an antibody forming cell or the generation of an antigen-specific memory B lymphocyte. Upon secondary contact with the same antigen an individual is capable to respond more rapidly through this memory B cell population, than it was able to do at the first contact with antigen. Also a shift in the predominancy of the class of antibodies produced, has been noted from IgM in the primary response to IgG in the secondary (memory) response. This is probably related to a shift in the class of immunoglobulin that is present on the precursors of the antibody producing cells, the B lymphocytes. Recently Coffman and Cohn (1977) demonstrated that two populations of virgin B cells exist that can respond to trinitrophenyl (TNP)-Ficoll. The first population of B cells has only IgM determinants and this subclass of cells can only produce IgM antibodies. The second set of cells carries both IgM and IgD on the surface and is capable to differentiate into IgM as well as IgG antibody forming cells. Hardly any IgG bearing cells can be found among virgin B lymphocytes. However, most memory precursors of IgG producing cells carry IgG on their surface. This is consistent with results reported by others (Strober, 1976b; Mason, 1976; Okumura et al., 1976) about the immunoglobulin class on the surface of primed B cells. Almost simultaneously similar results as obtained by Coffman and Cohn (1977) about the relation between the various classes of surface immunoglobulin on primed and non-primed B cells and the resulting classes of antibody forming cells have been described by Zan-Bar et al. (1977a; 1977b). The observations of these two groups that the appearance of IgG on B lymphocytes seems to be antigen-dependent, is contradictory to the model proposed by Cooper et al. (1972) in which they suggest an antigen-independent switch from IgM to IgG on B lymphocytes.

Besides the class of immunoglobulin also the density of surface immunoglobulins on B memory cells differs from virgin B lymphocytes. Both from direct evidence presented by Osmond and Nossal (1974a) and Strober (1975) and from indirect evidence presented by Klinman et al. (1973) and Klaus (1975) it can be concluded that the surface immunoglobulin density on B memory cells is higher than on virgin B lymphocytes. Perhaps, the higher receptor density on B memory cells is responsible for the high avidity of these cells as suggested by Klaus (1975). An increased average avidity of antigen receptors on B memory cells may also be involved, since memory cells are supposed to be a selection of precursors bearing high avidity receptors on their surface (Davie and Paul, 1972; Smith et al., 1974). Nevertheless, Möller et al. (1973) and Kim and Siskind (1974) presented evidence that also B memory cells with low avidity receptors can be demonstrated.

As discussed before B memory cells are supposed to differ in two more aspects from virgin B cells: their migrational behaviour and their lifespan. B memory cells are considered to be long-lived and have the ability to recirculate between lymph and blood.

As to the question where B memory cells are generated, there is compelling evidence that germinal centers in various organs are involved in the generation of B memory cells (Thorbecke et al., 1974; White, 1975). Nevertheless there is circumstantial evidence indicating that the generation of B memory cells can also occur outside germinal centers, probably in the primary follicles of the peripheral lymphoid organs (Rozing et al., 1977c).

The peripheral part of germinal centers contains "dendritic cells" (Veerman and Van Ewijk, 1975). These cells show large "dendritic" cytoplasmic extensions which are in close contact with surrounding mitotic lymphocytes and lymphoblasts in the germinal center. It has been suggested that this cell type plays an essential role in the generation of B memory cells (Van Ewijk, 1977). The following hypothesis about antigen-dependent B lymphocyte differentiation and proliferation is based on that assumption.

Under normal circumstances probably a constant and low amount of various antigens is present in an individual. These antigens induce a humoral immune response and will be phagocytized by polymorphs and macrophages or otherwise paralysed. It is also possible that complement factors attach to the antigen-antibody complex thereby producing antigen-antibody-complement complexes. Such complexes may circulate and attach themselves to dendritic cells that are present in primary follicles (Van Ewijk and Van der Kwast, 1977) through Fc or complement receptors on these cells, as supposed by Klaus and Humphrey (1977). Experiments performed by Veerman and Van Rooyen (1975) suggest that these complexes are carried towards dendritic cells by Fc receptors on B lymphocytes. However, C_3-depletion by cobra venom factor treatment prevents the antibody-dependent localization of antigen in splenic follicles (Papamichail et al., 1975; White et al., 1975; Klaus and Humphrey, 1977) indicating a more important role for complement and complement receptors in this process. These bound complexes on dendritic cells may play a role in the localization of B lymphocytes in follicles by providing passing lymphocytes the possibility to bind non-specifically through their own Fc or C_3 receptors. Since the number of virgin, specific antigen binding B cells is rather low, the chance that B cells specific for that particular antigen will bind to the complex is minimal. Such specific binding will presumably occur through the specific antigen receptor on the B cell, the surface immunoglobulin. The overall picture in this situation will be as follows: Migrating or recirculating B cells attach themselves non-specifically to antigen-antibody-complement complexes, which are bound to dendritic cells in follicles. Since the binding is not accomplished through the specific antigen receptor these B cells are not triggered to proliferate.

Another sequence of events may occur when the concentration of a particular antigen is significantly increased. In that case the presentation of the specific antigen on dendritic cells through antigen-antibody-complement complexes is much higher and the chance that specific B cells will encounter them is enormously enlarged. White (1975) describes such a system as a "trapping mechanism, whereby B lymphocytes of the appropriate specificity could be selected from the recirculating pool of

these cells". Upon interaction between the specific B cell through its antigen receptor and the complex a mutual stimulation between the triggered lymphocytes and the dendritic cell may result in a transformation of both cell types and thereby the formation of a germinal center. The extensions of dendritic cells in active germinal centers are indeed much more abundant than in primary follicles (Van Ewijk and Van der Kwast, 1977) thereby amplifying the efficiency of the system. As a result of the observed proliferation in germinal centers probably antigen-specific B memory cells are generated. Involvement of antigen-antibody-complement complexes and their contact with dendritic cells in the generation of B memory cells has been suggested recently by Klaus and Humphrey (1977) based on their observation that B memory cells fail to develop in mice which are depleted of circulating C_3 by continuous treatment with cobra venom factor.

Besides an antigen-specific B cell proliferation probably also the trapping of B cells with other specificities through their Fc and C_3 receptors continuously proceeds in germinal centers. These B cells may also be stimulated to proliferate in this environment. Germinal centers may therefore act as a "site at which nonspecific amplification of the B cell population can occur" as hypothesized by Davies and Carter (1972). Presumably B memory cells can also be generated in primary follicles although on a much lower scale.

It has been demonstrated by the failure of nude mice to develop germinal centers (Jacobson and Thorbecke, 1974) that germinal center formation is also T cell dependent. Weissman et al. (1976) have actually shown the presence of cells bearing T cell markers within germinal centers. The role of T lymphocytes in germinal center formation is unknown. The immune response to numerous antigens is T cell dependent. Possibly also the initial triggering of virgin B lymphocytes to proliferate resulting in the generation of B memory cells is T cell dependent or at least develops much more efficiently in the presence of T lymphocytes.

INTRODUCTION TO THE EXPERIMENTAL WORK

As described in the preceeding chapter substantial progress has recently been made in the field of the localization of B lymphocyte differentiation in mice. The major production of virgin B lymphocytes in mice seems to be located in the bone marrow. Furthermore spleen and germinal centers in peripheral lymphoid organs have been mentioned as sites for the amplification of antigen-inexperienced B lymphocytes. In spite of these investigations the significance of the various organs and structures for the overall B cell production is not well understood. The purpose of the experimental work described in the following papers has been to clarify the contribution of the various lymphoid organs to B cell differentiation in mice and the mutual relationship of these organs in that process.

In order to investigate B lymphocyte differentiation the recovery of the B cell population in mice depleted from their own B lymphocytes by a lethal dose of Röntgen-irradiation and reconstituted with syngeneic fetal liver cells of embryos at 14 days gestation has been used as a model system. Fetal liver cells have been chosen as a source of hemopoietic stem cells since at 14 days gestation no mature, surface immunoglobulin positive B lymphocytes were found in that organ. Therefore the B cells appearing in irradiated and reconstituted mice can be considered as the result of a new differentiation from immature precursor cells, probably hemopoietic stem cells, present in the fetal liver graft rather than the result of a proliferation of existing B cells.

The first four papers deal with the contribution of and the relationship between the various parts of the lymphoid system in the process of B cell differentiation. In the last paper experiments have been described to elucidate the histological substrate of B lymphocyte differentiation in the peripheral lymphoid organs.

Paper I is concerned with a study on the recovery of humoral immune responsiveness in mice after irradiation and reconstitution. In this study two aspects of the recovery of the capacity to respond to antigenic stimulation have been investigated: *a.* The time sequence of appearance of the two lymphoid cell types involved in the humoral immune response against thymus-dependent antigens, B and T lymphocytes; *b.* The contribution of the various lymphoid organs tested, i.e. spleen, bone marrow, peripheral and mesenteric lymph nodes and Peyer's patches, to the recovery of the B cell population. B lymphocytes in this study were determined by a morphological criterion, i.e. the presence of specific B cell surface receptors, the surface immunoglobulins, as well as a functional parameter, i.e. the ability to restore the adoptive immune response upon transfer to irradiated recipients. T lymphocytes were recognized through the expression of specific T cell markers on their surface, shown by an indirect immunofluorescence technique.

a. Within a week after irradiation and reconstitution a rapid increase of the number of immunoglobulin positive B lymphocytes was found in the various lymphoid organs resulting in a normal pre-irradiation level of B cell numbers at week 6. Also after approximately one week the first B lymphocytes that could be recognized functionally appears, followed by a continuous increase in the number of such cells. It took, however, somewhat longer for this cell population to reach normal cell numbers as compared with the B cell population defined by surface immunoglobulins. At 10 weeks after irradiation and reconstitution the functionally defined B cell population was restored quantitatively to a normal level. Comparison of the recovery pattern of surface immunoglobulin positive B lymphocytes and functionally reactive B cells suggests that immunoglobulin positive B lymphocytes are initially functionally not fully qualified, but gain this ability rapidly during further maturation. The appearance of another receptor on B lymphocytes, possibly the complement receptor, may be responsible for making the cell fully antigen-reactive. This suggestion would be in agreement with the results reported by Osmond (1976) and Ryser and Vassalli (1974) who showed that the C_3-receptor on B lymphocytes appears later than the surface immunoglobulins. Also in ontogeny and during the recovery after irradiation and bone marrow reconstitution such a sequential development of surface immunoglobulins and the C_3-receptor on B lymphocytes has been described (Gelfand et al., 1974).

The first appearing functional B cells were the precursors for IgM producing cells followed consecutively by the precursors of IgG and IgA producing cells. The sequence IgM-IgG-IgA found in this study corresponds well with the development of the B cell line in the chicken embryo (Kincade and Cooper, 1971), the human fetus (Lawton et al., 1972), adult thymectomized, irradiated and reconstituted mice (Van Muiswinkel et al., 1975) and patients recovering from a severe combined immunodeficiency disease after a successful thymus and bone marrow transplantation (Radl et al., 1971; Vossen et al., 1973).

As compared to the B cell compartment the recovery of the T cell population in the peripheral lymphoid organs was slower. Therefore the delay in recovery of humoral immune responsiveness against thymus dependent antigens after irradiation and reconstitution as reported by several authors (Aisenberg and Davis, 1968; Gregory and Lajtha, 1970; Rotter and Trainin, 1975) corresponds to the slow development of T lymphocytes rather than to the recovery of B lymphocytes.

b. By comparing the B cell recovery pattern of the various lymphoid organs after irradiation and reconstitution both Ig-positive and antigen-reactive B cells were found to appear in the spleen before they could be demonstrated in the other lymphoid organs. These results are compatible to those reported by Nossal and Pike (1973) and Gelfand and colleagues (1974) studying B cell differentiation in irradiated and reconstituted mice and during ontogeny. The interval between the appearance of B cells in the spleen and in other organs tested, however, was rather short in the latter studies. The time difference between the recovery of the B lymphocyte population in the spleen and that in the other organs tested was found to increase

when the irradiated mice were reconstituted with another source of hemopoietic stem cells that were not contaminated with mature B lymphocytes, i.e. spleen colony cells instead of fetal liver cells. This appeared to be caused by the number and nature of the reconstituting hemopoietic stem cells. It seems reasonable to conclude from the predominance of the spleen in the recovery of both immunoglobulin positive and antigen-reactive B lymphocytes after irradiation and reconstitution that the spleen is involved in the differentiation pathway of B lymphocytes. Whether the function of the spleen is an inductive one or just to provide an environment in which cells already committed to B cell differentiation can finish their further maturation remains a question.

In view of the predominant role of the spleen in the recovery of the B cell population after irradiation and reconstitution of normal mice, experiments were performed to test whether the contribution of the spleen is essential to B cell recovery. These experiments are described in *paper II.* Young adult mice were splenectomized two months before irradiation and reconstitution. In the bone marrow, lymph nodes and Peyer's patches of such mice still a recovery of both immunoglobulin positive and antigen-reactive B lymphocytes was found. As compared to sham-operated, irradiated and reconstituted control mice the recovery of the B cell population in splenectomized mice was not delayed nor did it result in lower numbers of B lymphocytes in the remaining organs. It can therefore be concluded from these experiments that the involvement of the spleen in B cell differentiation is not indispensable. The number of B lymphocytes in the lymphoid organs of splenectomized mice was even greater than in the same organs of sham-operated mice throughout the recovery period indicating a compensation by the other organs for the absence of the B cell population of the spleen. Apparently the most important role of the spleen in B cell differentiation in normal mice is to provide an environment in which cells already committed to the B cell line can mature. Obviously other organs can provide the same facilities to B cells for their maturation. Under normal conditions the main site of generation of B lymphocytes seems not to be located in the spleen, although a contribution of the spleen to B lymphocyte production remains possible.

The compensatory effect of the increased numbers of B cells in the various organs of splenectomized mice was also reflected in the level of the serum immunoglobulins. Apart from a lower IgM concentration in the serum of splenectomized mice no significant differences were found in the levels of the other immunoglobulin classes between splenectomized and sham-splenectomized mice.

Paper III deals with experiments about the influences of neonatal and adult splenectomy of non-irradiated mice on the humoral immune system. In this study essentially identical results were obtained as described for splenectomized, irradiated and reconstituted mice in paper II. In non-irradiated splenectomized mice also the number of B lymphocytes in the other lymphatic organs was increased. These B cells were fully antigen-reactive and the concentrations of serum immunoglobulins, except for the IgM class, were unaffected. The concentration of serum IgM was significantly lower in the blood of both neonatally and adult splenectomized mice,

than it was in sham-operated control mice. In order to locate the defect that was responsible for the low IgM concentration in the blood of splenectomized mice the various differentiation steps of the humoral immune system have been analyzed. In sham-operated mice, as is the case in normal mice (Haaijman, 1977), the majority of IgM-producing plasmacells was located in the spleen. The low concentration of IgM in the blood of splenectomized mice proved to be caused by a failure of the remaining organs to compensate completely for the removal of the population of IgM-producing plasmacells in the spleen. Obviously the number of precursors of IgM-producing cells in bone marrow and lymph nodes of splenectomized mice is increased and the capacity of these cells to differentiate into IgM-producing cells is not disturbed by splenectomy. Nevertheless the number of IgM-producing plasmacells in these organs remains the same after splenectomy. It seems therefore reasonable to conclude that especially the spleen provides a highly efficient environment for the specific differentiation step from precursor IgM-producing cell into IgM-producing cell.

In Paper II and III the influence of splenectomy on the synergistic abilities of bone marrow and thymus cells has also been investigated since neonatal as well as adult splenectomy and congenital absence of the spleen have been reported to lead to a failure of bone marrow and thymus cells to cooperate in the adoptive plaque forming cell response to sheep red blood cells (Bucsi et al., 1972; Wargon et al., 1975). However, no effect of either neonatal splenectomy or adult splenectomy followed by irradiation and reconstitution could be demonstrated on the synergistic abilities of these cells. It is concluded that the influences as described by Bucsi et al. (1972) and Wargon et al. (1975) are caused by a less sensitive test system, an explanation that also has been suggested by Auerbach and colleagues (1976).

Paper IV describes experiments performed to investigate the contribution of the bone marrow to B cell differentiation in irradiated and reconstituted mice. Therefore such mice were treated with high doses of strontium-89. ^{89}Sr is a bone-seeking radio-isotope which makes the marrow cavity a hostile environment for cell proliferation. In a dose of 3μCi ^{89}Sr/g body weight it causes a complete depletion of all nucleated cells of the bone marrow. In the spleen of irradiated and reconstituted mice with such an experimentally induced medullary aplasia still a recovery of the B cell population occurred. Only the number of B lymphocytes in the spleen never passed the 50% level as compared with non-^{89}Sr-treated control mice. These results are highly compatible to those reported by Kincade and coworkers (1975) working on B cell development in a similar system. Several explanations may be given for the lower numbers of B lymphocytes in the spleen of ^{89}Sr-treated mice: (1) Since hemopoiesis in such mice is only present in the spleen (Järplid, 1974) competition for stem cells may occur between the various cell lines generated in this organ and the stem cell pool may be quantitatively a limiting factor in the generation of B lymphocytes. (2) Although virgin B lymphocytes are supposed to be non-recirculating cells, it cannot be excluded that some B cells are eliminated while circulating in the blood through the marrow cavity. (3) However, the most important cause for

the 50% reduction of the number of B cells in the spleen of ^{89}Sr-treated mice may be the absence of a continuous transport of immature B lymphocytes from the bone marrow towards the spleen followed by a maturation of these cells into B cells in the spleen. It is suggested that in the bone marrow of normal irradiated and reconstituted mice an enormous production of B lymphocytes occurs identical to the process as described by Osmond (1975; 1976) for normal non-irradiated mice, followed by a migration of such newly formed cells to the spleen. A transport of newly formed cells from the bone marrow towards spleen and lymph nodes has been demonstrated in guinea pigs and rats by Brahim and Osmond (1970; 1973). This hypothetical transport would be absent in ^{89}Sr-treated irradiated and reconstituted mice.

The first conclusion that can be drawn from these experiments is that B cell differentiation in irradiated and reconstituted mice can occur outside the bone marrow, probably in the spleen and is in agreement with the statement of Phillips and Miller (1974) that "the bone marrow environment is not required for B cell differentiation". A second conclusion, however, drawn from the 50% reduction of the number of B cells in the spleen of ^{89}Sr treated mice may be that evidence is obtained for an important function of the bone marrow environment in the process of B cell differentiation in irradiated and reconstituted mice under normal circumstances.

Paper V deals with the histological substrate of the B cell recovery after irradiation and reconstitution. In order to study B lymphocyte recovery specifically B cells were determined in frozen sections of spleen, lymph nodes and Peyer's patches using a fluorescent anti-immunoglobulin serum as a staining reagent. The first B lymphocytes after irradiation and reconstitution appeared in the central area of the periarteriolar lymphatic sheath (PALS) in the spleen. They could be demonstrated dispersely, in the PALS over a period of two to three days before they started to organize themselves in the normal B cell compartments in the spleen, the primary follicles. Subsequently strongly fluorescent B cells, which may represent B memory cells, appeared in the marginal zone of the spleen, well before the development of germinal centers both in the spleen and other parts of the lymphoid system. It is suggested from this sequence that B memory cells may also be generated outside germinal centers, possibly in primary follicles.

The PALS in the spleen is a thymus-dependent area (Veerman and Van Ewijk, 1975) and it has been shown by several authors (Nieuwenhuis and Ford, 1976; Van Ewijk and Van der Kwast, 1977) that rather mature B lymphocytes migrate through the PALS on their way to their specific environment, the follicles. Probably the appearance of the first B lymphocytes after irradiation and reconstitution in the PALS reflects a similar migration pathway for newly formed B lymphocytes. This finding would then provide circumstantial evidence for a generation of B cells outside the spleen, probably in the bone marrow. Indeed, Brahim and Osmond (1970; 1973), investigating the migration of newly formed bone marrow lymphocytes, found that these cells were also located in thymus-dependent areas. These authors argued

that the non-specific homing pattern of newly formed cells might be due to the immaturity of such cells.

The time delay between the first appearance of B cells in the central part of the PALS and the formation of primary follicles may be caused by the fact that the first B lymphocytes appearing in the spleen have not yet developed receptors on their surface which are involved in the homing and retention of B cells in primary follicles. Perhaps this may be the same receptor as suggested in paper I for the development of antigen-reactivity: the complement receptor. A role for the complement receptor in the homing process of B lymphocytes has been proposed (see Antigen-dependent B cell differentiation).

B cell development in lymph nodes and Peyer's patches started somewhat later than in the spleen, but once started, structural recovery of the B cell compartment was completed very fast in these organs. It is suggested that rather mature B lymphocytes were involved in the B cell recovery in lymph nodes and Peyer's patches. These mature B lymphocytes were probably derived from the spleen. B cells in lymph nodes and Peyer's patches seemed also to migrate through thymus-dependent areas.

Based on the observations as reported in these papers the following hypothesis on B lymphocyte differentiation in lethally irradiated and reconstituted mice has been formed: In lethally irradiated and fetal liver reconstituted mice B lymphocytes are produced in the bone marrow. The first stages of B cell development proceed in the bone marrow. Once committed to the B cell lineage the development of characteristic B cell markers such as Fc receptors, surface immunoglobulins and complement receptors on these cells is primarily a matter of time (Osmond, 1976). Around the time that the B lymphocytes become immunoglobulin positive, they leave the bone marrow. The exact point of time for that event is not dependent on the stage of differentiation of the cell as determined by the presence of surface markers but is possibly related to the surface charge of the cell. Compatible with this suggestion is the observation of Osmond (1975) that during maturation B lymphocytes express a clear reduction of their negative surface charge. Such newly formed B lymphocytes migrate primarily to the spleen for further maturation. Within the spleen the normal B cell migration pathway through thymus dependent areas is followed. After their arrival in the spleen it takes some time before these B lymphocytes localize in primary follicles and gain full antigen-reactivity. Perhaps this delay reflects the time needed for the development of another receptor on the B cells after the appearance of surface immunoglobulins. It is suggested that the same receptor may be involved in both processes: the complement receptor. Indeed the experiments of Osmond (1975) and Ryser and Vassalli (1974) pointed out that the complement receptor on B lymphocytes appears in the spleen. From the spleen probably a migration of now mature B lymphocytes occurs towards lymph nodes and Peyer's patches. On their way to the primary follicles in these organs, B cells traverse again thymus-dependent areas.

From the extirpation and depletion studies it is obvious that in the process of

B cell differentiation in lethally irradiated and reconstituted mice no indispensable organ has been found. Compensation for the absence of certain parts of the hemopoietic and the lymphoid system seems to be a common phenomenon. Therefore probably alternative B cell differentiation and migration pathways may occur. In this hypothesis only the main outlines of B lymphocyte differentiation in irradiated and reconstituted mice have been indicated.

SUMMARY

An important contribution to the defense of the body against foreign invaders like bacteria and viruses is provided by the humoral immune system. Antibodies, the mediators of humoral immunity, are produced by highly specialized lymphoid cells, the plasmablasts and plasmacells. The progenitors of these antibody secreting cells are represented by a subpopulation of lymphoid cells: the B lymphocytes. The main objective of the first chapter of this thesis has been to emphasize the various characteristics of these B lymphocytes. Special attention has been paid to the differentiational aspects of B lymphocytes.

B cells are characterized by a number of specific properties: (1) B lymphocytes have specific membrane determinants. (2) They are localized in clearly outlined compartments in peripheral lymphoid organs. (3) They can be divided into a short-lived, non-recirculating, antigen-inexperienced (virgin) subset and a long-lived, recirculating antigen-experienced (memory) subpopulation. Antigen primed (memory) B cells are probably generated in germinal centers throughout the body. An attempt has been made to indicate the sequence of events occurring in these structures that lead to the generation of B memory cells.

This thesis has been focussed mainly on the virgin B cell subpopulation. A scheme of antigen-independent B cell differentiation has been drawn up that covers the various models proposed by other authors on that subject. In this scheme it is suggested that after the stage of commitment to the B cell lineage B cell differentiation is a gradual, autonomously regulated process in which specific B lymphocyte markers are expressed more or less sequentially.

It is obvious from the literature that at least the start of this B cell differentiation in normal mice is located in the bone marrow. Involvement in B lymphocyte differentiation of other organs has been proposed, but the magnitude of such a contribution is still unclear. The purpose of the experimental work described in the appendix papers of this thesis has been to gain more insight in the relationship between the various parts of the lymhpoid system in and their contribution to B cell differentiation. B cell differentiation has been investigated using the recovery of the B lymphocyte population in irradiated, fetal liver reconstituted mice as a model system.

Paper I describes experiments about the recovery of humoral immune responsiveness after irradiation and reconstitution. Two main aspects have been studied: (1) the time sequence of appearance of B and T lymphocytes, and (2) the contribution of the spleen, bone marrow, peripheral lymph nodes, and Peyer's patches to the recovery of the B cell population. It was concluded that the slow reappearance of immunological responsiveness to thymus-dependent antigens after irradiation and reconstitution as reported by others was related to the recovery pattern of the T cell population rather than of the B cell population. Individual B lymphocytes, developing after irradiation and reconstitution, seemed to lack antigen-reactivity at first, although they expressed already surface immunoglobulins. It is suggested

that this was caused by the initial absence of a specific membrane determinant, the complement receptor. Of the various organs tested, the spleen played the most predominant role in the process of B cell recovery after irradiation and reconstitution. Both immunoglobulin-positive and antigen-reactive B lymphocytes appeared in the spleen before they could be demonstrated in any other lymphoid organ. It was however not clear whether this role of the spleen was an inductive one or a supporting one.

Paper II describes the recovery of B lymphocytes in mice splenectomized before irradiation and reconstitution. In such mice the recovery of the B cell population as determined by both surface immunoglobulins and antigen-reactivity was not delayed nor diminished. The numbers of B lymphocytes in bone marrow, lymph nodes, Peyer's patches and blood of splenectomized mice were even higher than in the comparable organs of sham-operated control mice, indicating a compensatory mechanism regulating for the absence of the splenic B cell population. It is therefore concluded from these experiments that (1) the spleen is not essential for B cell differentiation and maturation after irradiation and reconstitution, and (2) the role of the spleen in the process of B cell differentiation in normal mice is probably not an inductive one.

Paper III reports experiments on the influence of neonatal and adult splenectomy of non-irradiated mice on the humoral immune system. In such treated mice also no essential role of the spleen in the early stages of B cell differentiation could be established. However trying to locate the defect in the humoral immune system responsible for the low IgM-concentration in the blood of splenectomized mice as observed in both paper II and paper III, indications were obtained that especially the spleen provides a highly efficient environment for the specific antigen-dependent differentiation steps from a presumable IgM-positive B lymphocyte into an IgM-producing plasma cell.

Paper IV describes the recovery of B lymphocytes in the spleen and bone marrow of irradiated and reconstituted mice in which the bone marrow had been depleted by ^{89}Sr-treatment. From the B cell recovery pattern in the spleen in the complete absence of a repopulation of the bone marrow it could be concluded that B cell differentiation after irradiation and reconstitution can occur outside the bone marrow. Nevertheless also circumstantial evidence was obtained from these experiments for an important inductive role of the bone marrow environment in normal irradiated and reconstituted mice.

Paper V reports a histological study on B cell recovery in the spleen, lymph nodes and Peyer's patches of irradiated and reconstituted mice using a specific staining technique. These experiments demonstrated that newly formed B cells like more mature B lymphocytes migrated through thymus dependent areas in the spleen towards their specific compartments. Furthermore indirect evidence was obtained from the observation that the first B lymphocytes in the spleen appeared in a specific migration environment that these cells were derived from outside the spleen, probably from the bone marrow. It is suggested that after the appearance

of surface immunoglobulins another determinant has to be expressed on the surface of B lymphocytes in order to facilitate the localization of B cells in primary follicles. For this function again the complement receptor on B lymphocytes has been mentioned. Finally it has been concluded from the delayed, but once started, rapidly proceeding B cell repopulation pattern in lymph nodes and Peyer's patches that the recovery of B lymphocytes after irradiation and reconstitution in these organs probably depends on a secondary migration of more mature B lymphocytes from the spleen towards lymph nodes and Peyer's patches.

Based on the data presented in these papers a hypothesis on antigen-independent B lymphocyte differentiation in lethally irradiated and reconstituted mice has been formed: In lethally irradiated and fetal liver reconstitued mice B lymphocytes are produced in the bone marrow. The early stages of B cell development are proceeding in the bone marrow. Around the time the cells become immunoglobulin positive they leave the bone marrow and migrate primarily to the spleen for further maturation. Within the spleen they follow a migration pathway which is identical to that of mature B lymphocytes. The time delay between the first arrival of B lymphocytes in the spleen on one hand and their localization in primary follicles and their acquisition of immune-reactivity on the other reflects the time needed for the development of a specific determinant on the cell surface, probably the complement receptor. From the spleen a migration of mature B lymphocytes occurs towards lymph nodes and Peyer's patches. Other B cell differentiation and migration pathways may occur, but they are probably of minor importance.

SAMENVATTING

Het humorale immuunsysteem neemt een belangrijk deel van de afweer tegen het lichaan binnendringende micro-organismen, zoals bacteriën en virussen voor zijn rekening. De factoren in het bloed die voor de humorale immuniteit zorgen, zijn de *"antilichamen"*. Antilichamen worden gemaakt door gespecialiseerde lymfoide cellen: de plasmablasten en plasmacellen. De voorlopers van deze antilichaamvormende cellen zijn bepaalde typen lymfocyten, de *B lymfocyten*. Het voornaamste doel van de inleidende hoofdstukken van dit proefschrift is geweest het uiteenzetten van de verschillende karakteristieken van deze B lymfocyten.

B cellen worden gekenmerkt door een aantal specifieke eigenschappen: (1) B lymfocyten bezitten specifieke determinanten op de celmembraan. (2) Zij zijn gelocaliseerd in duidelijk begrensde compartimenten in de perifere lymfoide organen. (3) Zij kunnen worden geclassificeerd als kort-levende, niet-recirculerende (*virgin*) B lymfocyten, die nog geen contact hebben gehad met antigeen en als lang-levende, recirculerende (*memory*) B lymfocyten, die al wel in contact zijn geweest met antigeen. Deze laatste B cel populatie ontstaat waarschijnlijk in kiemcentra in de lymfoide organen in het lichaam. Een mogelijke volgorde is aangegeven van de gebeurtenissen, die plaatsvinden in een dergelijke structuur en welke leiden tot het ontstaan van *memory* B lymfocyten.

Dit proefschrift heeft voornamelijk de populatie van de kortlevende, niet-recirculerende (*virgin*) B lymfocyten tot onderwerp gehad. Er is een schema voor de antigeen-onafhankelijke B cel differentiatie opgesteld waarin de verschillende uit de literatuur bekende modellen zijn opgenomen. Dit schema gaat uit van de veronderstelling dat de differentiatie van B lymfocyten na hun definitieve toetreding tot de B cel lijn geleidelijk en autonoom gereguleerd verloopt. In de loop van dit proces komen de verschillende specifieke B cel eigenschappen min of meer opeenvolgend tot expressie.

In de literatuur zijn aanwijzingen te vinden, dat ten minste het eerste gedeelte van de B cel differentiatie in normale muizen plaatsvindt in het beenmerg. Door verschillende auteurs is tevens aangegeven dat ook andere organen betrokken zijn bij dit differentiatieproces. De omvang van een dergelijke bijdrage is echter nog onduidelijk. Het verkrijgen van meer inzicht in de onderlinge samenhang van de verschillende delen van het lymfoide apparaat en hun bijdrage in het differentiatieproces, is het doel geweest van het experimentele werk, dat beschreven is in de bijgevoegde publicaties. In dit werk is B cel differentiatie bestudeerd door gebruik te maken van een model systeem, waarbij muizen bestraald en vervolgens gereconstitueerd werden met embryonale lever cellen.

Publicatie I beschrijft het herstel van het immunologisch apparaat na bestraling en reconstitutie. De twee belangrijkste aspecten, die zijn bestudeerd, zijn geweest: (1) De tijdsvolgorde van het verschijnen van T en B lymfocyten, en (2) de bijdrage van de milt, het beenmerg, de perifere lymfeklieren, en de plaques van Peyer in het herstel van de B cel populatie. Uit dit onderzoek werd geconcludeerd, dat de lang-

zaam terugkerende capaciteit om een immunologische reactie tegen thymus-afhankelijke antigenen te initieren, voornamelijk is gerelateerd aan het herstel patroon van de T cel populatie. Individuele B lymfocyten die verschenen na bestraling en reconstitutie bleken aanvankelijk niet in staat volledig op antigeen te reageren ondanks het feit dat zij al wel immuunglobuline positief waren. Dit gebrek werd mogelijk veroorzaakt door de aanvankelijke afwezigheid van een specifiek bestanddeel van de membraan, de complement receptor, bij jonge B cellen. De milt vervulde van de verschillende geteste organen de belangrijkste funktie in het proces van het B cel herstel na bestraling en reconstitutie. Zowel immuunglobuline positieve, als antigeen reactieve B lymphocyten verschenen in de milt voordat zij in de andere organen werden gevonden. Het was echter niet duidelijk of de inductie tot B cel differentiatie in de milt plaatsvond. Er dient rekening gehouden te worden met de mogelijkheid dat de milt slechts een omgeving verschaft, waarin de verdere maturatie van elders tot differentiatie geinduceerde B lymfocyten, gunstig kan verlopen.

Publicatie II beschrijft het herstel van B lymfocyten in muizen, waarvan de milt is verwijderd voor bestraling en reconstitutie. Het herstel van de B cel populatie in gesplenectomeerde muizen, zowel morfologisch als functioneel gekarakteriseerd, bleek niet vertraagd, noch verlaagd te zijn ten opzichte van dieren die nog wel over een milt beschikten. De aantallen B lymfocyten in het beenmerg, de lymfeklieren, de plaques van Peyer en het bloed van gesplenectomeerde muizen waren zelfs groter dan in de overeenkomstige organen van dieren, die een controle operatie hadden ondergaan. Klaarblijkelijk beschikken de gesplenectomeerde muizen over een compensatie mechanisme dat reguleert voor de afwezigheid van de B cel populatie in de milt. Er werd op grond van deze experimenten geconcludeerd, dat (1) de milt niet onvervangbaar is in het proces van B cel differentiatie en maturatie na bestraling en reconstitutie, (2) de belangrijkste functie van de milt in dit proces in normale muizen waarschijnlijk niet de inductie tot B cel differentiatie is.

Publicatie III behandelt het effect van splenectomie op het humorale immuunsysteem bij normale muizen. Splenectomie werd verricht kort na de geboorte, of op volwassen leeftijd. Bij deze muizen werd eveneens geen duidelijke invloed van de milt op de vroege stadia van het B cel differentiatie proces gevonden. De serum-IgM concentratie in gesplenectomeerde muizen bleek echter sterk verlaagd te zijn (zie ook publicatie II). Door na te gaan waar het defect was gelegen dat verantwoordelijk was voor deze lage serum-IgM concentratie, werden aanwijzingen verkregen, dat de milt wel degelijk een belangrijke invloed heeft op de antigeen-afhankelijke differentiatie van B lymfocyt tot plasmacel. Waarschijnlijk is in de milt een zeer efficiënte omgeving aanwezig voor de specifieke differentiatiestap van IgM-positieve B lymfocyt tot IgM producerende plasmacel.

Publicatie IV beschrijft het herstel van de B cel populatie in de milt en het beenmerg van bestraalde en gereconstitueerde muizen, waarbij het beenmerg was gedepleteerd door middel van een strontium-89 behandeling. Op grond van het B cel herstel in de milt van deze dieren, kon worden geconcludeerd, dat B cell differentiatie na bestraling en reconstitutie ook buiten het beenmerg kan plaatsvinden. Desalniettemin

werd uit deze experimenten ook indirect bewijsmateriaal verkregen voor de hypothese, dat in normale bestraalde en gereconstitueerde muizen het beenmerg een belangrijke rol speelt bij de inductie tot B cell differentiatie.

Publicatie V behandelt de histologische aspecten van het B cel herstel na bestraling en reconstitutie. Hiertoe werden milt, lymfeklieren en plaques van Peyer bestudeerd gebruik makend van een specifieke kleuringstechniek. Deze experimenten toonden aan dat pas gevormde B lymfocyten op hun weg naar hun specifieke compartimenten een migratieweg door de milt volgen welke identiek is aan die van rijpe B cellen, nl. door het thymus afhankelijke gedeelte van de milt. De waarneming, dat de eerste B lymfocyten in de milt verschenen in specifieke migratie kanalen, verschafte bovendien indirect een aanwijzing, dat deze cellen afkomstig zijn van buiten de milt, waarschijnlijk uit het beenmerg. Mogelijkerwijs moet na de verschijning van de immuunglobuline moleculen aan het oppervlak van de cel nog een andere membraan receptor tot expressie komen om de localisatie van B cellen in primaire follikels te bewerkstelligen. Mogelijk speelt ook hier de complement receptor een rol. Het B cel herstel in lymfeklieren en plaques van Peyer is vertraagd in vergelijking met de milt. De opbouw van de B cel populatie in deze organen is, eenmaal gestart, echter zeer snel volledig afgerond. Hieruit kan de conclusie worden getrokken, dat het herstel van het B cel compartiment in lymfeklieren en plaques van Peyer na bestraling en reconstitutie waarschijnlijk berust op een secundaire migratie van rijpere B lymfocyten vanuit de milt naar deze organen.

Gebaseerd op de gegevens, die gepresenteerd zijn in deze artikelen is een hypothese opgesteld over het verloop van de antigeen onafhankelijke B cel differentiatie in bestraalde en gereconstitueerde muizen. In lethaal bestraalde muizen die gereconstituteerd zijn met embryonale lever cellen, worden B lymfocyten geproduceerd in het beenmerg; de vroege stadia van de B cel ontwikkeling verlopen *in* het beenmerg. Rond het tijdstip dat deze cellen immuunglobuline positief worden verlaten zij het beenmerg en migreren voornamelijk naar de milt om daar verdere maturatie te ondergaan. Op hun weg door de milt volgen zij een migratieweg, welke identiek is aan die van volwassen B lymfocyten. De periode, die verstrijkt tussen de aankomst van de eerste immuunglobuline positieve B lymfocyten in de milt enerzijds en de lokalisatie van deze cellen in primaire follikels en hun uitrijping tot immuun reactieve cel anderzijds, weerspiegelt de tijd die nodig is voor het tot expressie komen van een specifieke membraan component, waarschijnlijk de complement receptor. Vanuit de milt vindt vervolgens een migratie van rijpe B lymfocyten plaats naar lymfeklieren en plaques van Peyer.

B cel differentiatie en migratie kunnen ook langs andere wegen verlopen. Deze zijn echter waarschijnlijk in normale muizen van minder belang.

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NAWOORD

Graag wil ik mijn dank betuigen aan allen, die direkt en indirekt hebben meegewerkt aan het tot standkomen van dit proefschrift. In het bijzonder ben ik mijn promotor Prof. Dr. O. Vos erkentelijk voor de mij geboden mogelijkheid om experimenteel onderzoek te verrichten en voor zijn kritische begeleiding bij zowel het experimentele werk alsmede het opschrijven van de verkregen resultaten in publikaties. Tevens wil ik hem ook dank zeggen voor zijn hulp bij het schrijven van de inleidende hoofdstukken van dit proefschrift. De beide co-referenten, Dr. W. Hijmans en Dr. L.D.F. Lameijer wil ik gaarne bedanken voor het kritisch doornemen van het manuscript van dit proefschrift.

René Brons ben ik dankbaar voor zijn nauwgezette, zeer zelfstandig uitgevoerde bijdrage aan het technische gedeelte van het experimentele werk en zijn kritische instelling bij het plannen van de verschillende experimenten, gedurende de tijd, dat wij hebben samengewerkt.

Mijn collega's van de groep Immunologie en Hematologie wil ik bedanken voor de waardevolle diskussies over alle mogelijke onderwerpen. Met name wil ik Rob Benner noemen. Zonder zijn enthousiaste steun zou het in dit proefschrift beschreven onderzoek niet de voorspoedige start hebben gehad, als nu het geval is geweest.

De verschillende medewerkers van het Instituut voor Experimentele Gerontologie te Rijswijk en het Medisch Biologisch Laboratorium te Rijswijk en vooral Dr. O. Brocades Zaalberg, wil ik dank zeggen voor hun waardevolle suggesties tijdens de woensdag-middag werkbesprekingen. Dr. W. Hijmans, Riek Schuit, Joost Haaijman en Jos Bloemmen van het Instituut voor Experimentele Gerontologie en Flora Lubbe van het Medisch Biologisch Laboratorium ben ik erkentelijk voor hun hulp bij het opzetten van de immunofluorescentie techniek in Rotterdam en de mogelijkheden, die zij mij ook daarna altijd hebben geboden bij het oplossen van problemen op dit gebied. Prof. Dr. L.M. van Putten van het Radio Biologisch Instituut te Rijswijk wil ik bedanken voor de mij geboden faciliteiten op zijn laboratorium om de strontium-89 experimenten uit te voeren. Joost Haaijman, Riek Schuit, Dr. J.M.N. Willers van de Rijksuniversiteit te Utrecht en Dr. O. Brocades Zaalberg ben ik dank verschuldigd voor het ter beschikking stellen van specifieke anti-sera. I am very obliged to Dr. I.L. Weissman, Stanford University, California, USA for introducing me in the field of fluorescence on frozen sections. I am also very grateful to him and Dr. S. Strober, Stanford University, California, USA for valuable discussions about B cell differentiation.

Ineke Leenders en Gerrit Kootte wil ik bedanken voor hun bijdrage aan het experimentele werk dat ze hebben geleverd gedurende hun keuzepraktikum en als studenten-assistent, tijdens hun medische opleiding aan de Erasmus Universiteit. Mejuffrouw P. van der Berg (Instituut voor Experimentele Gerontologie), de heer F. Luiten (Medisch Biologisch Laboratorium), Adrie van Oudenaren, Gerda van Pelt-van der Meulen, en Joke de Ruiter (Radio Biologisch Instituut) ben ik dankbaar voor alle technische hulp, die zij direkt en indirekt hebben verleend.

Leny Breteler, Inge Horssen-van der Kaai, Rita van den Hoek-Boucke, Cécile van Vugt-van Vliet, en vooral Cary Clerkx, bedank ik voor al het typewerk, dat zij in de loop van de tijd naast al hun andere bezigheden zo voortreffelijk hebben verzorgd. De heer J. Fengler en Tarry van Os wil ik gaarne bedanken voor al het fotografisch werk, dat zij voor mij hebben verricht. Margot van Duuren, Piet Hartwijk en Pim Visser dank ik voor het uitstekende tekenwerk van grafieken en figuren, en Mevrouw Godijn en Jopie Bolman voor het schone glaswerk en vooral de thee en koffie. Margot van Duuren en Rein Smid ben ik erkentelijk voor hun hulp bij het plaatsen van bestellingen van materiaal.

Joop Bos en Cornelie Franken wil ik bedanken voor de uitstekende verzorging van de proefdieren. Vooral bij bestralingsexperimenten zoals beschreven in dit proefschrift kan niet voldoende benadrukt worden hoezeer men afhankelijk is van een goede verzorging van deze dieren.

Prof. Dr. D. Bootsma, Hans Jongkind, Dick Klepper en Andries Westerveld ben ik erkentelijk voor de prettige en leerzame samenwerking in het Dagelijks Bestuur van onze vakgroep. Hetty, René, Rudy, Arthur en Dr. de Ruiter wil ik bedanken voor de fijne sfeer op ons lab.

Tenslotte dank ik mijn ouders, die het mij mogelijk maakten om te gaan studeren.

Antoinet, dank je wel voor *"being you"*.

CURRICULUM VITAE

Na het behalen van het diploma Gymnasium-B aan het Caland Lyceum te Rotterdam in 1967 begon ik in november van dat jaar met de studie Biologie aan de Rijksuniversiteit te Leiden. Het kandidaatsexamen werd afgelegd in november 1971 en het doctoraal examen in december 1973 (hoofdvakken dierfysiologie en immunologie).

Vanaf januari 1973 ben ik werkzaam geweest binnen de vakgroep Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam. Van januari tot december 1973 als doctoraal student biologie ter voorbereiding van mijn doctoraal examen en sedert juni 1974 als wetenschappelijk medewerker. Vanaf januari 1974 werd binnen de vakgroep Celbiologie en Genetica het in dit proefschrift beschreven onderzoek verricht.

Paper I

B Lymphocyte Differentiation in Lethally Irradiated and Reconstituted Mice. II. Recovery of Humoral Immune Responsiveness.

J. Rozing, N.H.C. Brons and R. Benner.

B-Lymphocyte Differentiation in Lethally Irradiated and Reconstituted Mice

II. Recovery of Humoral Immune Responsiveness

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Received July 7, 1976

The recovery of humoral immune responsiveness was studied in lethally irradiated, fetal liver-reconstituted mice. By means of both membrane fluorescence and antibody formation to sheep red blood cells (SRBC) as a functional assay, the rate of recovery of the compartments of B and T lymphocytes was determined in various lymphoid organs. The recovery of the immunoglobulin-positive (B) cell compartment after irradiation and reconstitution started in the spleen. This organ was also found to be the first in which the recovery of the B-cell population was completed. The interval between the recovery of the B-cell population in the spleen and that in the other organs tested was found to increase when the irradiated mice were reconstituted with spleen colony cells instead of fetal liver cells. This proved to be caused by the number and nature of the reconstituting hemopoietic stem cells. The immunoglobulin-positive (B) cells were found to appear before SRBC-reactive B cells could be demonstrated in spleen, lymph nodes, and Peyer's patches. The appearance of T lymphocytes in the various lymphoid organs required even more time. By means of cell transfer experiments, a sequential appearance of the precursors of anti-SRBC IgM-, IgG-, and IgA-plaque-forming cells could be demonstrated in spleen, bone marrow, lymph nodes, and Peyer's patches.

INTRODUCTION

Reappearance of immunological responsiveness after lethal irradiation and reconstitution with hemopoietic stem cells depends on the recovery of two types of lymphoid cells: B lymphocytes and T lymphocytes. Using functional tests, it was shown that the B-cell population recovers faster than the T-cell population (1–3). Comparison of the contribution of various lymphoid organs to the recovery of the B-cell population in irradiated and fetal liver-reconstituted mice revealed that the B-cell compartment of the spleen recovered somewhat earlier than the B-cell population in the other lymphoid organs tested (4–6). Osmond and Nossal (7, 8) and Osmond (9) presented evidence that in normal mice the bone marrow is the major site of lymphocyte production. These lymphocytes probably belong to the B-cell line (8). In our previous studies (6, 10), we presented arguments that after irradiation and reconstitution the bone marrow is also involved in the recovery of the B-cell population. However, using mice with a ^{89}Sr-induced bone marrow aplasia for irradiation and reconstitution, we found that the recovery of the B-cell compartment can also occur at extramedullary sites (10).

In our previous studies, B cells were assayed by means of membrane fluorescence using surface immunoglobulin (Ig) as a marker. Although surface immunoglobulins are considered to be the antigen receptors on B lymphocytes (11, 12), the appearance of Ig on B cells does not necessarily imply that these cells become immunocompetent. Using a functional B-cell assay, Van Muiswinkel *et al.* (13) demonstrated a rapid development of precursors of IgM-producing plaque-forming cells (IgM-PFC) in the spleen of irradiated and bone marrow-reconstituted mice, followed by a somewhat slower recovery of the precursors of IgG- and IgA-PFC. The fast appearance of precursors of IgM-PFC in that study was probably due to the reconstitution with bone marrow cells. It is known that the bone marrow of normal mice contains IgM B cells (3).

In the present study, the rate of development of the cell types involved in the recovery of humoral immune responsiveness was investigated in irradiated mice reconstituted with fetal liver cells as a B-cell free source of hemopoietic stem cells. The contribution of the various lymphoid organs to the recovery of the B-lymphocyte population is analysed using both surface-Ig and antibody formation to SRBC as B-cell markers.

MATERIALS AND METHODS

Animals

(C57BL/Rij × CBA/Rij) F1 female mice, 10–14 weeks old, were used. They were purchased from the Medical Biological Laboratory, Rijswijk, The Netherlands and from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands.

Preparation of Cell Suspensions

Mice were killed with ether. Immediately after killing, the organs to be used [fetal livers or spleens, femurs, peripheral lymph nodes (inguinal, brachial, and axillary), mesenteric lymph nodes, and Peyer's patches] were removed and brought into a balanced salt solution (BSS). This solution was prepared according to Mishell and Dutton (14). Spleen colonies were removed under a dissection microscope and pooled in BSS. Blood was obtained by cardiac puncture and immediately heparinized. Bone marrow was collected by flushing the femurs with BSS. Spleens, lymph nodes, Peyer's patches, bone marrow, fetal livers, and spleen colonies were minced with scissors and squeezed through a nylon-gauze filter to give a single cell suspension. Nucleated cells were counted with a Model B coulter counter (Coulter Electronics Ltd., Bedfordshire, England). Results obtained from the femoral marrow were extrapolated to the marrow present in the whole animal using the data of Chervenick *et al.* (15), who showed that, in mice, one femur contains 5.9% of the total bone marrow.

X Irradiation and Reconstitution

Recipient mice received 9^{25} rad of whole-body X irradiation, generated in a Philips Mueller MG 300 X-ray machine, as described in detail previously (10). Irradiated control mice died in 9–13 days.

To examine the recovery of the B-cell compartment, lethally irradiated mice were reconstituted with either 1.5×10^6 syngeneic fetal liver cells or a number of spleen-colony cells equivalent to three dissected spleen colonies.

Fetal livers were used from mice at 14 days of gestation. Until Day 17 of gestation, virtually no immunoglobulin-positive cells were found to be present in fetal liver (Table 1). Spleen colonies were obtained from spleens of lethally irradiated mice 12 days after intravenous (iv) injection of these mice with 10^4 normal bone marrow cells. Twelve-day spleen colonies did contain hemopoietic stem cells, but, in accordance with their erythropoietic, granulopoietic, or megakaryocytic nature (16, 17), no immunoglobulin-bearing lymphocytes were found (Table 1).

Isolation of Nucleated Cells from Peripheral Blood

Nucleated cells were isolated from mouse blood using a modification of the Ficoll-Isopaque system described by Böyum (18). The Ficoll-Isopaque mixture was obtained by mixing 2 parts Isopaque (Nyegaard and Co., Oslo, Norway), 3 parts 9% Ficoll (Pharmacia, Uppsala, Sweden), and 2 parts of a 3.5% solution of bovine serum albumin in aqua dest. The final density was 1.115 g/ml. Using this method, a minimum of 80% of the nucleated cells was recovered.

Spleen Colony Assay

The spleen colony assay of Till and McCulloch (19) was used to measure the number of colony forming units (CFU-S). The mice were killed on Day 8 or 9 after irradiation and the spleens were fixed in Telleysniczky's solution. The colonies were counted using a dissection microscope. Each experimental group consisted of 10–15 mice.

Immunofluorescence Staining of B and T Cells

Before reacting with antisera, the cells were washed three times in a solution consisting of 5% bovine albumin in phosphate-buffered saline (5% BA-PBS) (20).

For detection of B lymphocytes, a rhodamine-conjugated goat anti-mouse immunoglobulin serum (TRITC-GaM-Ig) (Nordic, Tilburg, The Netherlands) was used in a direct vital staining technique (10).

To detect T lymphocytes, an indirect immunofluorescence staining technique was used. Samples of 10^6 cells, in aliquots of about 25 μl, were added to 25 μl of a rabbit anti-mouse thymocyte serum (ATS) and this mixture was then incubated for 30 min at 4°C with gentle shaking every 10 min. The ATS was a gift from Dr. J. M. N. Willers (University of Utrecht, The Netherlands) and prepared as described by Veldkamp *et al.* (21). Both in our (22) hands and in the hands of Veldkamp *et al.* (21), this batch of ATS stained at least 98% of all thymocytes, less than 1% of the spleen cells, and 4% of the lymph node cells of adult thymectomized, lethally irradiated and bone marrow-reconstituted mice. These results using heterologous ATS in an indirect immunofluorescence technique agree well with the results of Raff and Owen (23) using a cytotoxicity test with an alloantiserum against the θ-determinant and with the data of Raff *et al.* (24) using an immunofluorescence technique. Furthermore, in a double-staining technique, no cells were found that stained with both the anti-mouse immunoglobulin serum and the ATS. After incubation with ATS, the cells were washed three times in 1% BA-PBS, resuspended in a final volume of 25 μl, and mixed with 25 μl of a

rhodamine-labeled goat anti-rabbit immunoglobulin serum (TRITC-GaR-Ig) (Nordic, Tilburg, The Netherlands). After incubating for another 30 min at 4°C with gentle shaking every 10 min, the cells were washed three times with 1% BA-PBS and mounted on a glass slide. As a control, cells were first incubated with normal rabbit serum or with 5% BA-PBS (instead of the ATS) and subsequently with the TRITC-GaR-Ig.

The slides were examined with a Zeiss fluorescence microscope equipped with a vertical illuminator IV/F and an Osram HBO 50 mercury lamp. Cells with the morphology of lymphocytes displaying a ring, a cap, or speckled peripheral fluorescence were scored as positive. Occasionally occurring dead cells showing a uniform fluorescence were not scored.

Functional B-Cell Assay

The maturing lymphocyte population was examined for its ability to give rise to anti-body-forming cells (a functional B-cell assay) by means of cell transfer experiments (Fig. 1). Nucleated cells (10^7) of the suspension to be tested for B-cell activity were mixed with 4×10^8 SRBC and injected iv into lethally irradiated recipient mice within 4 hr after irradiation. One hour after inoculation with the appropriate suspension and SRBC, the mice received an optimal dose of 10^7 dexamethasone-resistant thymocytes (DRT).

DRT were obtained from mice (5–6 weeks old) intraperitoneally (ip) injected with 30 mg/kg body weight of dexamethasone-21-phosphate (Merck and Co., Rathway, New Jersey) 2 days previously. DRT were added to exclude the possibility that the PFC responses were limited by a lack of helper T cells (25). Four days after irradiation and cell transfer, all mice were boosted ip with 4×10^8 SRBC. Each group consisted of five mice. The spleen of the recipients was removed on Day 7 and the number of IgM-, IgG-, and IgA-plaque-forming cells (PFC) was determined in the plaque assay. The number of PFC per spleen was considered to be an estimate of the number of B cells present in the cell suspension transferred 7 days before.

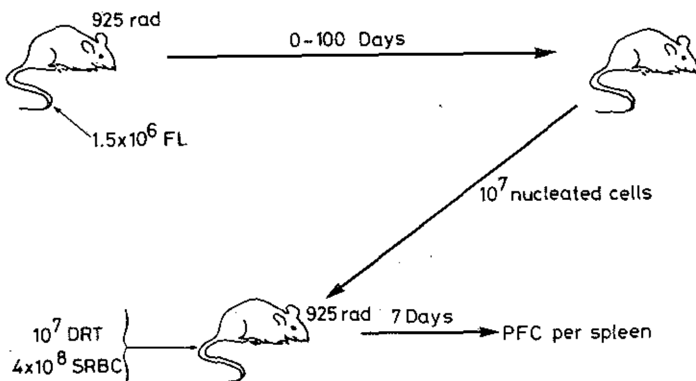

FIG. 1. The experimental procedure used to study development of the population of antigen-reactive B cells in mice after irradiation (925 rad) and reconstitution with 1.5×10^6 fetal liver cells (FL).

SRBC were obtained as a sterile suspension in Alsever's solution from the Department of Clinical Microbiology, Erasmus University, Rotterdam, The Netherlands. Before use, the cells were washed three times in PBS (pH 7.2).

Assay for PFC

The method for detection of PFC developed by Cunningham and Szenberg (26) was adopted with some modifications as described by Zaalberg *et al.* (27). One coverslip of 60 ×24 mm was used per microscope slide. The maximum concentration of the cell suspensions to be tested was 2×10^7 cells/ml and a volume of 0.3 ml was used for each test. The chambers were incubated at 37°C for 2 hr. The number of IgM-PFC was determined in a direct assay in which the slides were developed with guinea-pig complement (Flow Laboratories, Rockville, Maryland) only. The number of IgM-PFC + IgG-PFC was determined in an indirect assay in which the slides were developed with guinea-pig complement and a rabbit anti-mouse IgG. The number of IgG-PFC was calculated by subtracting the number of PFC obtained in the direct assay from that obtained in the indirect assay. The number of IgA-PFC was calculated in a similar way employing a rabbit anti-mouse IgA.

Statistics

The standard deviation (SD) associated with p plaques counted was calculated as the square root of $p + 0.004 \times p^2$. This formula was drawn up by Jerne for the agar plaque assay. For calculation of the 95% confidence limits, the formula $p \pm 2$ SD was used. The presence of IgG-PFC and IgA-PFC was considered to be significant when there was no overlap between the upper 95% confidence limit in the direct assay and the lower 95% confidence limit in the indirect assay.

RESULTS

Recovery of the Ig-Positive B-Cell Compartment after Irradiation and Reconstitution with Fetal Liver Cells

To study the contribution of the various parts of the lymphoid system to the recovery of the B-cell population after irradiation and reconstitution, mice were lethally irradiated and reconstituted with 1.5×10^6 fetal liver cells. At various times after irradiation and reconstitution, total cellularity and numbers of B cells were determined in spleen, bone marrow, Peyer's patches, and peripheral lymph nodes of these mice. As shown in Fig. 2, a continuous decrease in number of nucleated cells was found in all the organs tested until 4 days after irradiation and reconstitution. After that time, a fast recovery of the cellularity could be demonstrated in spleen and bone marrow, resulting in normal cell numbers at about Day 13 and Day 22, respectively. A much slower repopulation was found in Peyer's patches and lymph nodes. These organs reached normal cell numbers not before Day 42.

After irradiation and reconstitution, the first significant increase of the number of Ig-positive (B) cells was found in the spleen at Day 11 (Fig. 3). Within 3 days after the reappearance of B cells in the spleen, the presence of Ig-positive

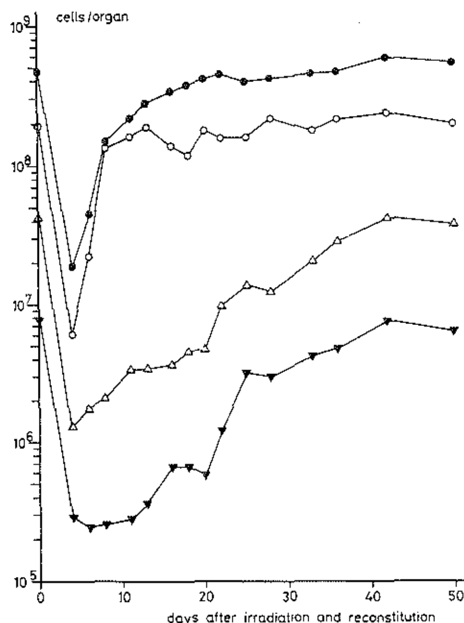

FIG. 2. The recovery of the number of nucleated cells in spleen (○), bone marrow (●), Peyer's patches (△), and peripheral lymph nodes (▼) of lethally irradiated, fetal liver-reconstituted mice. For each value, the organs of at least five mice were pooled.

cells could be demonstrated in the bone marrow. The number of B cells in both spleen and bone marrow rose very rapidly. This resulted in normal B-cell levels at Day 28 for the spleen and at Day 36 for the bone marrow. The (B-cell) repopulation of Peyer's patches and lymph nodes started somewhat later and occurred at a slower rate than in spleen and bone marrow. B-cell numbers in these organs reached the normal level at about Day 42.

The recovery pattern after reconstitution with fetal liver cells proved to be dependent on the dose of fetal liver cells used for reconstitution. Irradiated mice reconstituted with 3×10^6 fetal liver cells (Fig. 4) showed a similar repopulation pattern of the cellularity in spleen and bone marrow as described above (Fig. 2). A fast recovery starting at Day 3 resulted in normal cell numbers in spleen and bone marrow at Day 20. When irradiated mice were reconstituted with only 3×10^5 fetal liver cells, a delay in the repopulation of spleen and bone marrow was found. This resulted in a significant lower number of nucleated cells in the spleen before Day 13. From Day 13 until Day 20, the cellularity of the spleen of mice reconstituted with 3×10^5 fetal liver cells exceeded significantly the number of cells in the spleen of mice reconstituted with 3×10^6 fetal liver cells. After Day 20, no differences could be demonstrated between the splenic cell numbers of these two groups of mice. As far as the bone marrow is concerned, mice reconstituted with 3×10^5 fetal liver cells showed a prominent delay in the recovery of cellularity as compared with mice reconstituted with 3×10^6 fetal liver cells. Up to Day 30, the number of nucleated cells in the bone marrow of these mice was significantly lower than that of mice reconstituted with 3×10^6 fetal liver cells. After that time, no

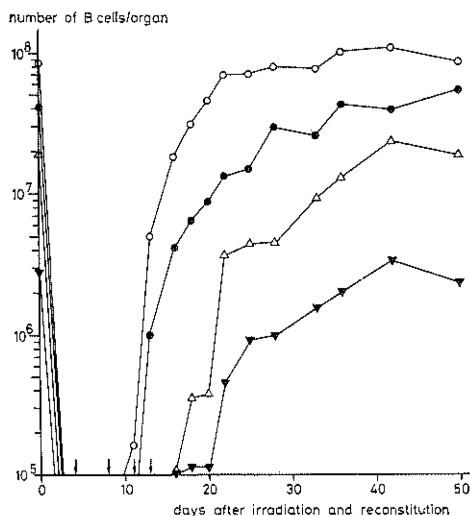

FIG. 3. The recovery of the number of Ig-positive (B) cells in spleen (○), bone marrow (●), Peyer's patches (△), and peripheral lymph nodes (▼) of lethally irradiated, fetal liver-reconstituted mice. For each value, the organs of at least five mice were pooled. Data were obtained from the same mice used to determine the recovery of the number of nucleated cells (Fig. 2). Arrows indicate cell numbers above background level but below the abscissa.

differences were found in the marrow cellularity between these two groups of mice. Probably the above-mentioned overshoot of splenic cellularity between Day 13 and Day 20 in the group of mice reconstituted with 3×10^5 fetal liver cells

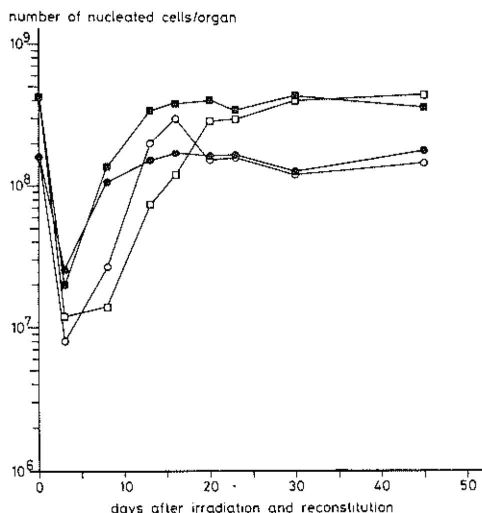

FIG. 4. The recovery of the number of nucleated cells in the spleen (circles) and bone marrow (squares) of lethally irradiated mice reconstituted with different doses of fetal liver cells. Irradiated mice were reconstituted with 3×10^5 fetal liver cells (open symbols) or 3×10^6 fetal liver cells (closed symbols). For each value, at least five mice were individually tested and the average is expressed.

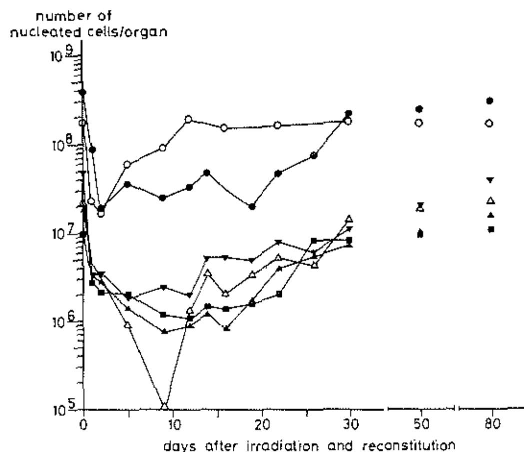

FIG. 5. The recovery of the number of nucleated cells in the spleen (○), bone marrow (●), mesenteric lymph node (▼), Peyer's patches (△), peripheral lymph nodes (▲), and peripheral blood (■) of lethally irradiated mice reconstituted with spleen colony cells. For each value, the organs of at least five mice were pooled.

compensated for the relatively low cell number and consequently reduced hemopoietic activity in the marrow during the first 3 weeks after irradiation and reconstitution.

Reconstitution with Spleen Colony Cells

The recovery of the Ig-positive cell population was also investigated in irradiated recipients reconstituted with spleen colony cells as a source of hemopoietic stem cells. These spleen colonies were dissected from the spleens of mice 12 days after irradiation and reconstitution with 10^4 normal bone marrow cells. Such colonies did contain hemopoietic stem cells, but no Ig-bearing lymphocytes could be found (Table 1). A number of spleen colony cells equivalent to three dissected spleen colonies were iv injected into secondary irradiated recipients. In the various lymphoid organs of these mice, the total number of nucleated cells and the number of B cells were determined at various times after irradiation and reconstitution. The organs tested were spleen, bone marrow, peripheral lymph nodes, mesenteric lymph node, Peyer's patches, and peripheral blood. After the initial decrease in the cellularity of all of the organs tested, a rapid increase in the number of nucleated cells was found in the spleen, starting between Day 2 and Day 5 and resulting in a normal cellularity in this organ at Day 12 (Fig. 5). In all of the other organs, including the bone marrow, a long-lasting severe depletion of the nucleated cells was found. Normal or nearly normal cell numbers were reached between Day 50 and Day 80. Only in the peripheral blood was the normal value found at Day 26.

The recovery of the B-cell population in mice reconstituted with spleen colony cells (Fig. 6) followed a pattern similar to the recovery pattern of the total number of nucleated cells (Fig. 5). After the initial decrease, the start of the recovery of the B-cell compartment began in the spleen between Day 5 and Day 8, resulting in normal numbers of B cells at Day 30. In the other organs, the recovery of the B-

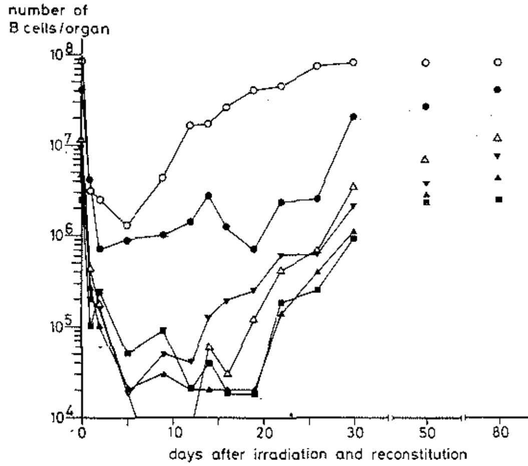

FIG. 6. The recovery of the number of Ig-positive (B) cells in the spleen (○), bone marrow (●), mesenteric lymph node (▼), Peyer's patches (△), peripheral lymph nodes (▲), and peripheral blood (■) of lethally irradiated mice reconstituted with spleen colony cells. For each value, the organs of at least five mice were pooled. Data were obtained from the same mice used to determine the recovery of the number of nucleated cells (Fig. 5).

cell population started between Day 15 and Day 20. In these organs, the recovery of the B-cell compartment was completed between Day 50 and Day 80.

As was found during the experiments in which the mice were reconstituted with fetal liver cells, the recovery pattern after reconstitution with spleen colony cells appeared to be dependent on the number of reconstituting cells (Fig. 7). Using a

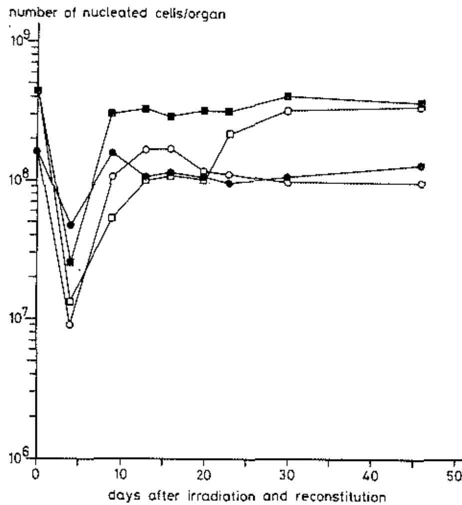

FIG. 7. The recovery of the number of nucleated cells in the spleen (circles) ad bone marrow (squares) of lethally irradiated mice reconstituted with different doses of spleen colony cells. Irradiated mice were reconstituted with a number of cells equivalent to 3 (open symbols) or 30 spleen colonies (closed symbols). For each value, at least five mice were individually tested and the average is expressed.

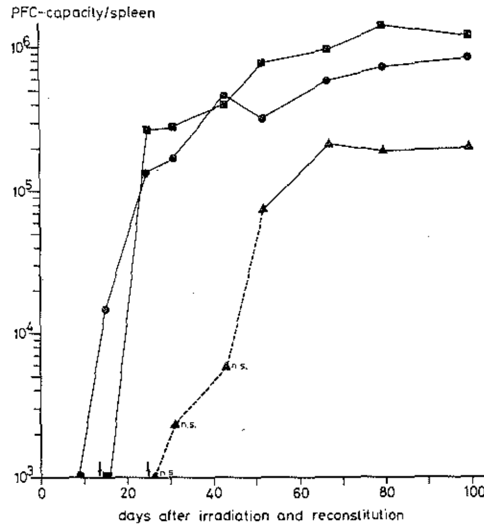

FIG. 8. The recovery of the number of SRBC-specific B cells in the spleen of irradiated and fetal liver-reconstituted mice. At various times after irradiation, the spleens of five reconstituted mice were removed and 10^7 nucleated cells of the pooled spleens were injected into secondary irradiated recipients together with 4×10^8 SRBC and 10^7 DRT. After 7 days, the number of IgM-, IgG-, and IgA-PFC per spleen was determined in the pooled spleens of four or five recipients. IgM- (●), IgG- (■), and IgA- (▲) PFC capacity (for definition, see Results) was calculated from the number of IgM-, IgG-, and IgA-PFC per recipient spleen and the total number of nucleated cells in the spleen of the donor mice. n.s. means that the number of plaques counted in the indirect assay (IgM- + IgA-PFC) did not differ significantly from the number of plaques counted in the direct assay (IgM-PFC). Arrows indicate cell numbers above background level but below the abscissa.

higher dose of spleen colony cells for reconstitution, i.e., a number of cells equivalent to 30 dissected spleen colonies, a recovery pattern was obtained similar to that using a high dose of fetal liver cells (cf. Fig. 4). After the initial decrease, a fast recovery of the cellularity was found in both spleen and bone marrow. Up to 46 days after irradiation and reconstitution in the bone marrow of mice reconsti-

TABLE 1

CFU-S and Ig-Positive (B) Cells in Fetal Livers and Spleen Colonies

Source of cells	Day	Number of nucleated cells ($\times 10^{-6}$)	CFU-S/10^5 nucleated cells[a]	Percentage of B cells[b]
Fetal livers	14[c]	23.0	5.8 ± 0.4	<0.01
	16	24.4	9.5 ± 0.7	<0.01
	17	22.1	11.6 ± 0.5	<0.01
Spleen colonies	12[d]	4.6	4.4 ± 0.2	<0.01

[a] Average ± 1 SEM.
[b] Ig-positive (B) cells were calculated as a percentage of all nucleated cells.
[c] Number of days after conception.
[d] Number of days after irradiation and bone marrow transplantation.

tuted with 30 spleen colonies, a significantly higher number of nucleated cells was found than in the bone marrow of mice reconstituted with 3 spleen colonies. Comparing Figs. 4 and 7, it should be noted that 3 × 10^5 fetal liver cells contain less CFU-S than do three spleen colonies (Table 1).

Recovery of the Antigen-Reactive B-Cell Population after Irradiation and Reconstitution

Subsequent to studies on the recovery of the Ig-positive (B) cell population by means of membrane fluorescence, the recovery of the capacity to give a PFC response after adoptive transfer with SRBC and a nonlimiting number of T cells was studied. This is a functional parameter for B-cell differentiation in lethally irradiated and reconstituted mice. Cell suspensions of organs to be tested for B-cell activity were transferred from irradiated, reconstituted mice to secondary irradiated recipients (Fig. 1). The spleens of the recipients were removed on Day 7 and the number of IgM-, IgG-, and IgA-PFC was determined with the plaque assay. To describe the amount of reactive B cells of an organ quantitatively, the conception "PFC capacity" was adopted from Benner *et al.* (28). These authors introduced and defined this conception as

$$\frac{\text{number of cells in the organ}}{\text{number of cells transferred into the recipient}} \times \text{mean number of PFC per recipient spleen.}$$

The recovery of the IgM-, IgG-, and IgA-PFC capacity in the spleen after irradiation and reconstitution is shown in Fig. 8. At Day 9 after irradiation and reconstitution, the first significant numbers of IgM-B cells could be demonstrated, since spleen cells transferred at that time evoked an IgM-PFC response in the spleen of the secondary recipient. Thereafter, the number of IgM-B cells increased, reaching a plateau level after Day 43. The first significant numbers of IgG-B cells were found at Day 12. Between Day 12 and Day 25, the number of IgG-B cells increased enormously. From Day 25 on, a higher number of IgG-B cells than of IgM-B cells could be demonstrated. No significant numbers of IgA-B cells were found before Day 52. This was probably due to the indirect plaque assay used for determination of IgA-PFC. Rather shortly after that time, the number of IgA-B cells also reached a constant level. A similar recovery pattern of the IgM-, IgG-, and IgA-PFC capacity was found in bone marrow, lymph nodes, and Peyer's patches (data not shown).

By adding the data of IgM-, IgG-, and IgA-PFC capacity, the total PFC capacity of the various lymphoid organs could be calculated. Comparing the recovery of the SRBC-specific B-cell compartment in these organs, the spleen was again found to be the first organ in which these B cells appeared (Fig. 9). In the spleen, the first significant numbers of SRBC-reactive B cells could be demonstrated on Day 9. From Day 9 up to Day 25, a logarithmic increase was found. After Day 25, the number of SRBC-reactive B cells increased gradually up to Day 67. At that time, a constant level was reached. The first B cells in the bone marrow and in the other organs tested, lymph nodes and Peyer's patches, could be demonstrated at Day 17 and Day 27, respectively. All of these organs showed a fast increase until Day 31. Thereafter, a weak but continuous rise was found, resulting in a constant level of the number of SRBC-reactive B cells in these organs at about Day 80.

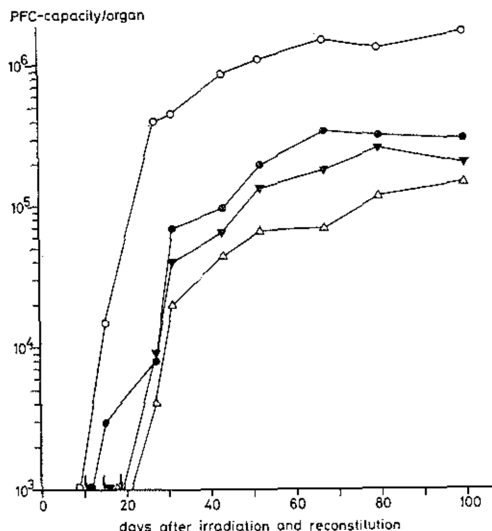

FIG. 9. The recovery of the number of SRBC-specific B cells in spleen (○), bone marrow (●), lymph nodes (▼), and Peyer's patches (△) of irradiated and fetal liver-reconstituted mice. At various times after irradiation, the lymphoid organs of five reconstituted mice were removed and 10^7 nucleated cells of the pooled organs were injected into secondary irradiated recipients together with 4×10^8 SRBC and 10^7 DRT. After 7 days, the number of IgM-, IgG-, and IgA-PFC per spleen was determined in the pooled spleens of four or five recipients. From the numbers of PFC per recipient spleen and the total number of nucleated cells in the lymphoid organs of the donor mice, the PFC capacity (for definition, see Results) was calculated. The total PFC capacity of the various organs was calculated by adding the data of IgM-, IgG-, and IgA-PFC capacity. Arrows indicate cell numbers above background level but below the abscissa.

Recovery of the T-Cell Population after Irradiation and Reconstitution

The recovery of the T-cell compartment was investigated in irradiated, fetal liver-reconstituted mice. T cells were assayed by membrane fluorescence using an antimouse thymocyte serum (ATS) in an indirect procedure. Due to the radiation sensitivity of T lymphocytes, a sharp decrease in the numbers of T cells was found in thymus, spleen, lymph nodes, and Peyer's patches after irradiation (Fig. 10). The first organ showing repopulation with T cells was the thymus. At Day 10, a fast recovery of the number of T cells started in this organ, an overshoot was found at Day 42, and a constant level was achieved from Day 72 onward. In the other organs tested, the recovery also started on Day 10, but initially at a much lower rate. The number of T lymphocytes in these organs reached a normal or nearly normal level between 88 and 200 days after irradiation and reconstitution. This delay can be attributed to the distribution of T cells from the primary organ, the thymus, to the secondary lymphoid organs, i.e., spleen, lymph nodes, and Peyer's patches.

DISCUSSION

The recovery of immune reactivity after irradiation and reconstitution occurs rather slowly. Rotter and Trainin (29) demonstrated that, up to 70 days after

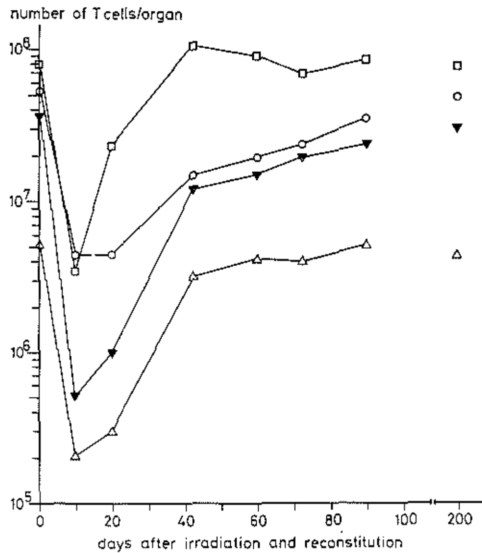

FIG. 10. The recovery of the number of T cells in thymus (□), spleen (○), lymph nodes (▼), and Peyer's patches (△) of lethally irradiated fetal liver-reconstituted mice. For each value, the organs of at least five mice were pooled.

irradiation and bone marrow reconstitution, the response to SRBC was seriously reduced in syngeneic chimeric mice. Working with a similar system, Gregory and Lajtha (2) determined the recovery of anti-SRBC responsiveness. In their study, PFC responsiveness was not detected for almost 2 weeks after irradiation and bone marrow grafting. This was followed by a rapid increase during the next 2 weeks and subsequently by a much slower recovery. At 70 days after irradiation and reconstitution, the PFC response to SRBC was still less than 50% of the normal value. These results correspond well with those reported by Aisenberg and Davis (30), who investigated the recovery of the hemolysin response in syngeneic chimeric CBA mice. Studying the recovery of the response to a conjugate of dinitrophenol and the flagella of *Salmonella adelaide* in the spleen of irradiated, fetal liver-reconstituted mice, Nossal and Pike (5) found a 30–80% restoration by 3–9 weeks after irradiation and transfusion. In these studies, the recovery of immunological responsiveness was investigated by determining the number of direct (IgM) plaque-forming cells against the respective antigens in the spleens of the irradiated and reconstituted mice. Stanković, *et al.* (31) also studied the recovery of immune reactivity by measuring the titre of agglutinating antibodies in the serum of irradiated mice treated with isogeneic bone marrow upon antigenic stimulation. Comparing the recovery of the antibody titre to SRBC and killed *Salmonella typhimurium* cells, they found that antibody responsiveness to *S. typhimurium* recovered to the normal value during the fourth week after irradiation and reconstitution, while the capacity of anti-SRBC antibody formation developed rather slowly and remained below the normal level during the experimental period, i.e., 80 days.

These differences may have been caused by a number of factors. Several authors described that hemopoietic stem cells (HSC) from various sources differ in their proliferative capacity, mitotic activity, and seeding efficiency. Comparing

fetal liver stem cells and bone marrow stem cells, Löwenberg (32) assessed the proliferative capacity of stem cells by determining the increase in CFU-S in the spleen and in the bone marrow after transplantation. The proliferative capacity of fetal liver HSC and bone marrow HSC differed significantly. The mean population doubling time of fetal liver CFU-S was 26 hr, whereas that of bone marrow CFU-S was 32 hr. Using a thymidine suicide technique, Becker *et al.* (33) presented evidence suggesting that, in normal adult bone marrow, only a few CFU-S are in cycle. In fetal liver, they found that a high proportion of CFU-S was in active cell cycle. In this paper, a clear effect of the source and number of HSC on the recovery pattern of both the total number of nucleated cells and the Ig-positive cell compartment was found. Probably the differences in repopulation capacity between spleen colony HSC and fetal liver HSC are caused by the fact that, when spleen colony cells are used for reconstitution, a secondary transplantation is performed. As shown by Vos and Dolmans (34), the proliferative capacity of secondary transplanted HSC is enormously lowered as compared with HSC obtained from normal bone marrow and spleen. However, by injecting a larger dose of these HSC, a repopulation pattern could be achieved that is similar to that obtained by using the more rapidly proliferating fetal liver HSC. Therefore, the period of recovery probably can be influenced by varying the number and nature of the HSC used for reconstitution.

The development of humoral immune reactivity after irradiation and reconstitution is related to the recovery of the three cooperating cell types involved in the humoral immune response against thymus-dependent antigens: B lymphocytes, T lymphocytes, and macrophages. Although macrophages are found to be radioresistant (35), the progenitors of macrophages, the promonocyte and the monocyte (36), are radiosensitive (35). Therefore, it cannot be excluded that the slow recovery pattern is caused by a radiation effect on these latter cell types. However, Kincade *et al.* (37) showed that, within a few weeks after radiation and reconstitution, the number of monocytes in the peripheral blood reaches the normal level. Since both B and T lymphocytes and their progenitors are radiosensitive (38, 39), the velocity of the recovery of one of these cell populations in quantitative or qualitative terms probably limits the development of the overall immune reactivity. By means of mebrane fluorescence, we found that the recovery of the Ig-positive cell population after irradiation and fetal liver reconstitution is completed before the T-cell population reaches its normal level (cf. Figs. 3 and 10). The recovery pattern of the Ig-positive cell compartment in this study agrees with that in comparable studies reported in the literature (4, 5). Although the B-cell compartment is restored quantitatively at Day 42 (Fig. 3), it takes more time to regain a normal population of Ag-reactive B cells. Possibly the appearance of another receptor on the lymphocyte membrane is required for the activation of B cells to antibody formation (40). Perhaps complement (41) or Fc receptors (42) on the B-lymphocyte surface are involved in this process. In ontogeny and during the recovery after irradiation and bone marrow reconstitution, a sequential development of the Ig receptor and the C3 receptor on B lymphocytes has been described (43, 44).

Our data suggest that the development of precursors of IgM-PFC corresponds to the recovery of the Ig-positive cell population. Uhr and Vitetta (45) described that the majority of B lymphocytes in mice carries IgM on their surface. If this is also true for B cells which appear after irradiation and reconstitution, the re-

covery of Ig-positive cells is largely due to the development of IgM-positive cells. According to the two-stage model for the development of antibody-producing cells as proposed by Cooper *et al.* (46), lymphocytes with IgM on their surface can differentiate into IgM-producing plasma cells. The appearance of precursors of IgG-PFC (and precursors of IgA-PFC) possibly reflects the differentiation of virgin IgM-bearing lymphocytes into IgG- (and IgA-) bearing memory cells (47). These latter cells can differentiate into IgG- (and IgA-) PFC after appropriate antigenic stimulation. This differentiation into memory cells might be caused by the continuous antigenic pressure of the environment (28, 48). The sequence IgM–IgG–IgA found in this study corresponds well to the development of the B-cell line in the chicken embryo (49), the human fetus (50), adult thymectomized, irradiated, and bone marrow-reconstituted mice (13), and patients recovering from a severe combined immuno-deficiency after a successful thymus and bone marrow transplantation (51, 52).

Comparing the contribution of the various lymphoid organs to the recovery of the B-cell population after irradiation and reconstitution, both Ig-positive and SRBC-specific B cells were found to appear in the spleen before they could be demonstrated in the other lymphoid organs. From experiments reported in a previous paper (10) and from the results of Phillips and Miller (53) and of Kincade *et al.* (37) using irradiated and reconstituted mice with an ^{89}Sr-induced medullary aplasia, it can be concluded that B-cell differentiation indeed can occur outside the marrow. This extramedullary differentiation process, however, does not result in normal B-cell numbers (10, 37). In normal mice, the bone marrow is the major site of lymphocyte production (7, 54). The newly formed lymphocytes are probably immature stages of the B-cell line (8). Since ^{89}Sr treatment of irradiated and reconstituted mice leads to the generation of subnormal numbers of B cells, the development of the B-cell population in such mice also seems to be bone marrow dependent. There probably is a continuous migration of immature Ig-negative precursor B lymphocytes from the bone marrow to the spleen (55). Shortly after the arrival of these cells in the spleen, they may acquire increasing amounts of surface Ig and become immunocompetent. Comparing the functional capacity of bone marrow lymphocytes with that of B lymphocytes from the spleen, Stocker *et al.* (56) found that the spleen is threefold more active per typical B lymphocyte as judged by surface Ig density than is the bone marrow.

Recently, Strober (57) described that splenectomy of adult rats did not affect the number of rapidly turning over virgin B cells present in thoracic duct lymph. In a similar approach with irradiated, fetal liver-reconstituted mice, we found that splenectomy could not prevent the appearance of B cells in the various lymphoid organs (6, 58). Therefore, we concluded that B cells can also mature in other organs than spleen. In conclusion, the appearance of Ig-positive cells in a peripheral lymphoid organ may be due to *in situ* maturation of Ig-negative precursor B cells immigrated from the bone marrow and to an influx of Ig-positive cells derived from other lymphoid organs.

The development of T-cell function after irradiation and reconstitution has been demonstrated by several authors (1–3) to be relatively slow. Gregory and Lajtha (2) suggested that the IgM response to SRBC in irradiated and bone marrow-reconstituted mice during the recovery period is limited by the number of T helper cells. In this paper, the recovery of the T-cell population after irradiation and re-

constitution has been quantitated in various organs by using immunofluorescence. The delay in recovery of T-cell function, as reported in the literature, proved to correspond well to the slow development of T-lymphocyte numbers found in this study.

It is of interest to compare the rate of recovery of the T-cell population in lethally irradiated (925 rad), reconstituted mice (this study) and in T-cell-deprived mice on which a nonirradiated neonate thymus (30) or a 300-rad-irradiated neonate thymus (30, 59) had been transplanted under the kidney capsule. In mice subjected to a lethal dose of X irradiation, the T-cell population seems to recover more slowly than in the other groups of mice. This may be attributed to damage of the thymic epithelium by high doses of irradiation. Investigating the thymus in isogeneic chimeras, Van Bekkum (60) indeed found that the number of thymic epithelial cells of mice irradiated with doses of over 800 rad decreased significantly. In addition, Aisenberg and Davis (30) and Davis and Cole (61) presented evidence that the inductive function of the thymic stroma is detectably damaged by irradiation doses of about 1 krad.

ACKNOWLEDGMENTS

The authors are very indebted to Professor Dr. O. Vos for helpful advise and criticisms during the performance of this study. Thanks are also expressed to Dr. J. M. N. Willers (University of Utrecht, The Netherlands) for the generous gift of the rabbit anti-mouse thymocyte serum (ATS) and the rabbit anti-mouse- IgA serum and to Dr. O. B. Zaalberg (Medical Biological Laboratory TNO, Rijswijk, The Netherlands) for supplying the rabbit anti-mouse IgG serum. The authors also express their thanks to Mrs. C. M. Leenders for excellent technical assistance.

This investigation is part of a project program on the regulation of hemopoiesis, subsidized by the Netherlands Foundation for Medical Research (FUNGO). J. Rozing was supported by the Interuniversitary Institute of Radiation Pathology and Radiation Protection (Leiden, The Netherlands).

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Paper II

B Lymphocyte Differentiation in Lethally Irradiated and Reconstituted Mice. III. The Influence of Splenectomy on the Recovery of the B Cell Population.

J. Rozing, N.H.C. Brons and R. Benner.

B Lymphocyte Differentiation in Lethally Irradiated and Reconstituted Mice

III. The Influence of Splenectomy on the Recovery of the B Cell Population

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The recovery of the B cell population was studied in irradiated and fetal liver reconstituted mice. Since in irradiated and reconstituted mice the B cell population in the spleen recovers much faster than in the other lymphoid organs, we assessed the role of the spleen in the recovery of the B cell compartment in the other organs. It was found that the absence of the spleen did not delay, nor diminish the recovery of the immunoglobulin-(Ig)-bearing (B) cell population in the bone marrow, lymph nodes, Peyer's patches and peripheral blood. Throughout the recovery period the number of B lymphocytes in the lymphoid organs of splenectomized mice was even greater than in the same organs of sham-operated mice. B cells obtained from the bone marrow of splenectomized, irradiated and reconstituted mice appeared to be fully immunocompetent, as shown by their ability to cooperate with thymocytes in an adoptive plaque-forming cell response to sheep red blood cells. The compensatory effect of the increased numbers of B cells in the bone marrow and peripheral lymphoid organs of splenectomized mice was reflected in the level of the serum immunoglobulins. Apart from a lower IgM concentration in the serum of splenectomized mice no significant differences were found in IgG_1, IgG_{2b} and IgA levels between splenectomized and sham-splenectomized mice. It is concluded that the spleen is not essential for both normal B lymphocyte differentiation and maturation after irradiation and reconstitution.

INTRODUCTION

By comparing the contribution of the various lymphoid organs to the recovery of the B cell compartment in irradiated and fetal liver reconstituted mice, it has been found that the B cell population in the spleen recovered earlier than the B cell population in the other lymphoid organs tested (1, 2). In these studies B cells were assayed by means of both membrane fluorescence using surface-immunoglobulin (Ig) as a marker (1, 2), and antibody formation to sheep red blood cells (SRBC) as a functional B cell test (2). Furthermore, evidence was presented that after irradiation and reconstitution the bone marrow is involved in the recovery of the B cell population (3, 4). On the other hand, using mice with a ^{89}Sr-induced bone marrow aplasia it was found that the recovery of the B cell compartment could also occur at extra medullary sites, e.g. the spleen (3-5).

Absence of the spleen markedly influences the immune system of both animals and man. Adult splenectomy affects among other things the serum-immunoglobulin levels, in particular of the IgM-class (6, 7) and evokes a long-lasting leucocytosis,

predominantly consisting of lymphocytes (8). Furthermore, neonatal splenectomy and congenital absence of the spleen have been reported to lead to an increased susceptibility for severe bacterial infections later on in life (9, 10) and, as has been reported for mice, to a failure of bone marrow and thymus cells to cooperate in the adoptive immune response against SRBC (11, 12). There are, however, little or no data in the literature about the role of the spleen in the early differentiation steps of the B cell line. Nevertheless the spleen may play an essential role in the B lymphocyte differentiation, especially during the recovery after irradiation and reconstitution, since the first appearing B cells are always located in the spleen (1-4).

In the present study the contribution of the spleen to the recovery of the B cell population after irradiation and reconstitution has been investigated. This was analyzed using both surface-Ig and adoptive antibody formation to SRBC as B cell markers.

MATERIALS AND METHODS

Animals. (C57BL/Rij x CBA/Rij) F1 female mice, 10-14 weeks old, were used. They were purchased from the Medical Biological Laboratory, Rijswijk, The Netherlands and from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands.

Splenectomy. Splenectomy and sham-splenectomy were performed as described previously (13) at least 1 month before irradiation and reconstitution. There was no post-operative mortality.

X-irradiation and reconstitution. Recipient mice received 925 rad whole body X-irradiation, generated in a Philips Mueller MG 300 X-ray machine, as described in detail previously (4). Irradiated control mice died in 9-13 days.

Lethally irradiated mice were reconstituted with 1.5×10^6 syngeneic fetal liver cells. These cells were injected intravenously (i.v.) within 2 hours after irradiation. Fetal livers were obtained from mice at 14 days of gestation. At that time virtually no immunoglobulin-bearing cells are present in fetal liver (less than 1 per 10^4 nucleated cells).

Preparation of cell suspensions. Cell suspensions of spleen, bone marrow, lymph nodes (inguinal, brachial, axillary, and mesenteric), Peyer's patches, thymus and blood were prepared in a balanced salt solution (BSS) as described previously (2). Nucleated cells were counted with a Coulter Counter Model B. Results obtained from the femoral marrow were extrapolated to the marrow present in the whole animal using the data of Chervenick, Boggs, Marsh, Cartwright and Wintrobe (14), who showed, that in mice one femur contains 5.9 per cent of the total bone marrow. Comparison of the percentages B cells in femur (8.7 ± 1.0), tibia (8.6 ± 0.6), humerus (9.3 ± 1.0), rib (8.3 ± 0.6) and sternum (9.9 ± 1.1) indicated that the B cell distribution in various compartments of mouse bone marrow is about the same.

Immunofluorescence staining of B cells. For detection of B lymphocytes a rhodamine conjugated goat anti-mouse immunoglobulin serum (TRITC-GaM-Ig) (Nordic,

Tilburg, The Netherlands) was used in a direct vital staining technique, which is described in detail in a previous paper (4).

Slides were examined with a Zeiss fluorescence microscope equipped with a vertical illuminator IV/F and an Osram HBO 50 mercury lamp. Cells with the morphology of lymphocytes displaying a ring, a cap or speckled peripheral fluorescence were scored as positive. Dead cells, which occurred occasionally, showing an uniform fluorescence were not scored.

Functional B cell assay. B lymphocytes in the bone marrow were examined for their ability to give rise to antibody-forming cells (a functional B cell assay) by means of cell transfer experiments. Irradiated recipients were i.v. injected with the appropriate cell suspension and 4×10^8 SRBC within 4 hr after irradiation. Each group consisted of 5 mice. On the fourth day all mice were boosted with 4×10^8 SRBC i.p. The spleen of the recipients was removed on day 7 and the number of IgM-plaque-forming cells (PFC) was determined in the plaque assay as described previously (2).

Serum immunoglobulins. Serum immunoglobulin concentrations (IgM, IgG_1, IgG_{2b}, IgA) were kindly determined by Mrs P. van der Berg (Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands) using the radial immunodiffusion technique (15).

RESULTS

Recovery of the Ig-positive cell population. In order to study the role of the spleen in the recovery of the B cell population after irradiation and reconstitution, splenectomized and sham-splenectomized mice were lethally irradiated and reconstituted with 1.5×10^6 fetal liver cells. At various times after irradiation and reconstitution the number of B cells was determined in the various lymphoid organs of these mice.

The recovery of the Ig-positive (B) cell population in splenectomized mice after irradiation and reconstitution showed that the first significant increase of the number of B cells occurred in the bone marrow at day 12. Such an increase could subsequently be demonstrated in the lymph nodes at day 15 and in Peyer's patches at day 17 (Fig. 1). The recovery of B cells in these three organs of splenectomized mice is somewhat faster and leads to higher numbers of B cells than in the same organs of sham-splenectomized mice. This phenomenon was especially evident after day 35. When mice were tested individually at 100 days after irradiation and reconstitution the number of B cells in the various organs of splenectomized mice proved to be significantly higher than in sham-splenectomized mice (Table I). This phenomenon was most prominent in the bone marrow, lymph nodes and blood.

Functional B cell assay. Neonatal splenectomy and congenital absence of the spleen are reported to lead to a decreased capacity of bone marrow and thymus cells to cooperate in adoptive PFC responses to SRBC (11, 12). These results suggest that in the newborn mouse, the spleen plays an important role in the appearance of fully immunocompetent B and T lymphocytes in the bone marrow and thymus, respectively. This led us to investigate, whether the spleen plays a similar role in lethally irradiated and

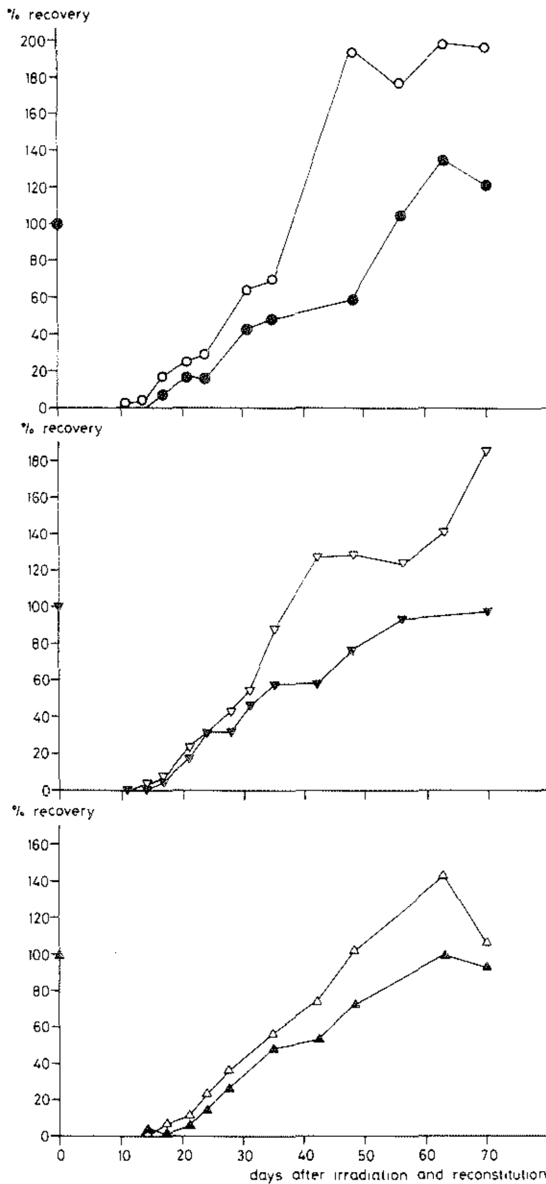

Fig. 1. The recovery of the number of Ig-positive (B) cells in the bone marrow (○, ●), lymph nodes (inguinal, brachial, axillary and mesenteric) (▼, ▽) and Peyer's patches (▲, △) of lethally irradiated, fetal liver reconstituted mice. The mice were splenectomized (open symbols) or sham-splenectomized (closed symbols) at least 1 month before irradiation and reconstitution. The numbers of B cells in an organ are expressed as percentages of the numbers of B cells present in the same organs of normal mice. For each figure at least five mice were used.

TABLE I

Number of B Cells in Various Organs of Mice Splenectomized or Sham-Splenectomized before Irradiation and Reconstitution

Organ[a]	number of B cells per organ x 10^{-7}	
	Sx	ShSx
spleen	–	9.08 ± 0.45[b]
bone marrow[c]	8.15 ± 0.54	4.05 ± 0.41
Peyer's patches	1.44 ± 0.18	1.01 ± 0.09
lymph nodes[d]	2.74 ± 0.20	0.98 ± 0.15
blood[e]	1.74 ± 0.06	0.63 ± 0.05

[a]Organs were removed from mice at 100 days after irradiation and reconstitution.
[b]Average ± 1 S.E.M. Each group consisted of 5 mice.
[c]Results obtained from the femoral marrow were extrapolated to the marrow present in the whole animal (see Materials and Methods).
[d]Inguinal, brachial, axillary and mesenteric lymph nodes were used.
[e]Total blood volume was estimated at 2 ml.

reconstituted mice. Therefore, the adoptive PFC response was determined of bone marrow cells and thymocytes from splenectomized and sham-splenectomized mice, irradiated and reconstituted 100 days previously.

Table II shows that 2 x 10^7 bone marrow cells from splenectomized mice transferred in combination with 2 x 10^7 thymocytes from sham-splenectomized mice evoked an increased number of PFC against SRBC as compared to 2 x 10^7 bone

TABLE II

Adoptive PFC Response to SRBC of Bone Marrow Cells of Mice Splenectomized or Sham-Splenectomized before Irradiation and Reconstitution

Experimental group[a]	IgM-PFC/spleen[b]
BM^{Sx}	3,088[c] (2,102 – 4,536)
BM^{Sx} + TH^{ShSx}	22,156 (16,595 – 29,580)
BM^{Sx} + TH^{Sx}	31,288 (27,662 – 35,388)
BM^{ShSx}	1,283 (742 – 2,220)
BM^{ShSx} + TH^{ShSx}	5,902 (4,299 – 8,135)
BM^{ShSx} + TH^{Sx}	5,015 (4,002 – 6,284)

[a] 2 x 10^7 Bone marrow cells (BM) together with or without 2 x 10^7 thymocytes (TH) of mice splenectomized (Sx) or sham-splenectomized (ShSx) before irradiation and reconstitution were transferred into irradiated recipients together with 4 x 10^8 SRBC. Samples were taken from mice at 100 days irradiation and reconstitution.
[b] Direct PFC-assay was done on the spleen of the recipient mice 7 days after cell transfer.
[c] Geometric mean and 95 per cent confidence limits. Each group consisted of at least 5 mice.

marrow cells from splenectomized mice transferred alone. The same number of bone marrow cells and thymocytes from splenectomized mice evoked a similar increase of the number of anti-SRBC PFC. No significant differences could be demonstrated between these two groups. From these results it can be concluded that after irradiation and reconstitution the spleen is not essential in establishing a good bone marrow-thymus synergism in the adoptive PFC response to SRBC.

The number of anti-SRBC PFC in the spleen of recipient mice, inoculated with bone marrow cells from splenectomized mice and thymocytes, was significantly higher than in the spleen of recipient mice, injected with bone marrow cells from sham-splenectomized mice and thymocytes. Apparently, the number of SRBC-reactive B cells in the marrow of splenectomized mice, is higher than in the marrow of sham-splenectomized mice, which is in analogy with the number of Ig-positive cells.

Serum-Ig levels. In order to investigate whether the increased numbers of Ig-positive cells and antigen-reactive cells in the lymphoid organs of splenectomized, irradiated and reconstituted mice could account for a compensatory mechanism, resulting in normal humoral immune responsiveness, serum-immunoglobulin levels were determined in the blood of these mice at 100 days after irradiation and reconstitution. By comparing the immunoglobulin concentrations in the blood of normal mice and mice, splenectomized or sham-splenectomized before irradiation and reconstitution, no significant differences were found for IgG_1 (Table III). A somewhat lowered IgG_{2b} concentration and an elevated IgA concentration was found in the serum of irradiated and reconstituted mice as compared with normal control mice. This may be due to the irradiation and reconstitution procedure, or merely to age differences. Splenectomy had no effect on the IgG_{2b} and IgA level. The only clear effect of splenectomy on serum immunoglobulin levels occurred in the IgM class.

TABLE III

Immunoglobulin Concentrations in the Blood of Mice Splenectomized or Sham-Splenectomized before Irradiation and Reconstitution

experimental group	immunoglobulin class			
	IgG_1	IgG_{2b}	IgM	IgA
normal mice[a]	0.98 ± 0.08[c]	0.65 ± 0.03	0.35 ± 0.02	0.41 ± 0.04
splenectomized mice[b]	0.99 ± 0.13	0.51 ± 0.03	0.19 ± 0.02	0.87 ± 0.03
sham-splenectomized mice[b]	1.18 ± 0.09	0.55 ± 0.04	0.42 ± 0.05	1.09 ± 0.13

[a] Normal non-irradiated mice, 12 weeks old.

[b] Immunoglobulin concentrations in the blood of splenectomized and sham-splenectomized mice were determined at 100 days after irradiation and reconstitution.

[c] Immunoglobulin concentrations (± 1 S.E.M.) in mg protein per ml. Each group consisted of at least 8 mice.

While no difference was found between the IgM level in normal mice and sham-splenectomized, irradiated and reconstituted mice, the IgM concentration in the blood of the splenectomized, irradiated and reconstituted mice was dramatically lowered as compared to both other groups of mice.

DISCUSSION

In normal mice the bone marrow is probably the main source of B lymphocytes (16, 17). These lymphocytes are initially immunoglobulin negative, but acquire an increasing amount of surface immunoglobulin during maturation (18, 19). This maturation process can take place in the bone marrow (17) but, as has been demonstrated in ^{89}Sr-treated mice, it can also occur in peripheral lymphoid organs (3-5). In guinea pigs and rats a continuous transport of newly formed lymphocytes from the bone marrow towards the spleen and lymph nodes has been found (20). Therefore it is very likely that also in normal mice B cell maturation can take place in other lymphoid organs than the bone marrow, e.g. peripheral lymphoid organs. Especially the spleen seems to be of importance for B cells to become antigen reactive (21, 22).

From decreased numbers of B cells found in lymphoid organs of irradiated and reconstituted mice with an ^{89}Sr-induced medullary aplasia it may be concluded, that the development of the B cell compartment after irradiation and reconstitution is also bone marrow dependent (3, 4). By comparing the contribution of the various lymphoid organs to the recovery of the B cell population in normal irradiated and reconstituted mice both immunoglobulin-positive and antigen reactive B cells could be demonstrated in the spleen before they appeared in the bone marrow (1, 2). This phenomenon may be caused by a migration of immature Ig-negative precursor B lymphocytes from the bone marrow to the spleen in these animals. We hypothesized that shortly after arrival in the spleen these cells acquired increasing amounts of surface-immunoglobulin and became immunocompetent (2). From the results of Strober (23), who demonstrated in normal adult rats that the number of rapidly turning over virgin B cells in the thoracic duct was not affected by splenectomy, it can be concluded that the other peripheral lymphoid organs can replace the spleen in this process. The present experiments using splenectomized mice suggest that this is also true for irradiated and reconstituted mice. Since B lymphocytes in splenectomized mice proved to be also fully immunocompetent (Table II), it can be concluded that the predominant role of the spleen in B cell development after irradiation and reconstitution is not an essential one. Possibly a common structure in lymphoid organs like the follicular system in spleen and lymph nodes is involved in the generation of B lymphocytes, as has been hypothesized by Nieuwenhuis and Keuning (24).

Comparing the recovery of the B cell compartment in the various lymphoid organs of splenectomized and sham-splenectomized mice a clear compensation for the absence of the B cell compartment of the spleen was found in the other lymphoid organs of the splenectomized animals. Especially in the bone marrow the number of B lymphocytes was increased (Table I). Probably a factor or a combination of

factors exists regulating the total number of B lymphocytes in an individual. Similar homeostatic influences have been described for the total number of hemopoietic stem cells (25) and the total amount of splenic tissue in an animal (26). A regulating influence of the spleen upon the overall cell proliferation in the bone marrow after irradiation has been stated (27). Therefore, the increase of the numbers of B cells in the bone marrow and in the peripheral lymphoid organs in splenectomized mice may be caused by the removal of a splenic factor regulating B lymphopoiesis in the bone marrow. Another explanation for the higher number of Ig-positive lymphocytes in the bone marrow of splenectomized, irradiated and reconstituted mice could be that the number of B memory cells in this organ is enlarged because the spleen is absent and consequently not able to sequester memory cells, seeding from the lymph nodes. An indication in this direction might be the fact that, as compared with sham-splenectomized mice, the increase of the adoptive PFC response to SRBC in the bone marrow of splenectomized mice was 4-6 fold (Table II), while only a 2 fold increase of the number of Ig-positive cells could be demonstrated in the marrow of these mice (Table I).

Since no significant differences were found in the amounts of immunoglobulins of the various classes between splenectomized and sham-splenectomized mice, except for the IgM-class, the compensation for the absence of the spleen in splenectomized mice, as found for Ig-positive and SRBC-reactive B cells, is also expressed in the endproduct of the B cell line, the serum immunoglobulins. The decrease of the IgM-level, as found in splenectomized animals (Table III), has also been demonstrated in patients after splenectomy (6) and in spleenless animals (7). Since the majority of IgM-synthesizing plasma cells in normal mice are located in the spleen (28), this fall in IgM concentration may reflect a failure of the remaining organs to compensate for the absence of splenic IgM plasma cells.

Battisto and co-workers (11) and Lozzio and Wargon (12) presented evidence that bone marrow and thymus cells obtained from congenital spleenless or neonatally splenectomized mice were defective in their capacity to cooperate in antibody formation after transfer into lethally irradiated recipients. These authors argue that the spleen plays an essential role in the development of both immunocompetent B and T lymphocytes in the bone marrow and thymus, respectively. There is a striking similarity between the circumstances under which lymphocytes develop in neonatally splenectomized or congenital spleenless animals and in splenectomized, irradiated and reconstituted mice. Nevertheless no lack of bone marrow-thymus synergism was found in these latter mice (Table II). Thefore our results are in accordance with the recent report of Landahl and co-workers (29), who showed that neonatal splenectomy and congenital absence of the spleen did not significantly reduce the capacity of bone marrow and thymus cells to cooperate in the adoptive PFC response against SRBC.

ACKNOWLEDGMENTS

The authors are very indebted to Professor Dr. O. Vos, Dr. O.B. Zaalberg and Dr. W. Hijmans for their helpful advise and valuable criticisms during the performance of this study. Thanks are also expressed to Mrs. P. van der Berg for determining serum immunoglobulin concentrations and to Mr. A. van Oudenaren and Mr. G. Kootte for excellent technical assistance.

This investigation is part of a project program on the regulation of hemopoiesis, subsidized by the Netherlands Foundation for Medical Research (FUNGO). J. Rozing was supported by the Interuniversitary Institute for Radiation Pathology and Radiation Protection (Leiden, The Netherlands).

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Paper III

Effects of Splenectomy on the Humoral Immune System. A Study in Neonatally and Adult Splenectomized Mice.

J. Rozing, N.H.C. Brons and R. Benner.

Effects of Splenectomy on the Humoral Immune System

A Study in Neonatally and Adult Splenectomized Mice

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Experiments were performed to investigate the influence of neonatal and adult splenectomy on humoral immunity in mice. In the bone marrow and lymph nodes of both groups of splenectomized mice the number of immunoglobulin (Ig)-positive (B) lymphocytes was significantly higher than in sham-operated mice. These B lymphocytes proved to be fully capable to differentiate into IgM- and IgG-producing cells. The higher number of B cells in the bone marrow and lymph nodes of splenectomized mice probably reflects a compensation for the absence of the B cell population of the spleen, since hardly any differences in the endproduct of the B cell line, the serum immunoglobulins, were found between splenectomized and sham-splenectomized mice. Only for the IgM-class a significant lower concentration occurred in the serum of splenectomized animals. In sham-operated animals the majority of IgM-producing plasmacells was located in the spleen. After splenectomy the number of IgM-producing plasmacells in bone marrow and lymph nodes was slightly higher. However, in contrast to all Ig-containing plasmacells, the total number of IgM-producing plasmacells was much lower in splenectomized mice than in sham-splenectomized animals. Therefore, the low concentration of IgM in the blood of splenectomized mice was caused by a failure of the remaining organs to compensate completely for the removal of the population of IgM-producing plasmacells in the spleen. Since in bone marrow and lymph nodes the number of precursors of IgM-producing plasmacells, the IgM-positive B lymphocytes, and their ability to differentiate into IgM-producing plasmacells, was not affected by splenectomy, possibly the differentiation from IgM-positive B cell into IgM-producing plasmacell is sub-optimally regulated in these organs. Obviously especially the spleen provides a highly efficient environment for this specific differentiation step.

The synergistic ability of bone marrow cells and thymus cells from neonatally splenectomized mice to cooperate in the adoptive plaque-forming cell response to sheep red blood cells (SRBC) was also investigated. Bone marrow cells and thymus cells of these mice were found to be fully capable to cooperate in the humoral immune response to SRBC.

INTRODUCTION

It is remarkable that an individual can survive the removal of an important organ as the spleen. In mice the spleen has in addition to its involvement in the regulation of the circulation of blood and the production of cells of the erythroid and granuloid series, an important role in the immune defense, especially against blood borne viral and bacterial infections (1). The fact, that splenectomy nevertheless in general does not cause any severe post-operative complications, suggests the existence in the body of strong compensatory mechanisms for the loss of the various functions of the spleen.

However, there have been reported various effects of removal of the spleen. These influences are mostly related to functions of the spleen in the immune system. Although no effect of splenectomy can be demonstrated on the primary allograft rejection (2, 3), the hyperacute secondary allograft rejection as found in secondary kidney transplantation in man, can be abolished by splenectomy before or during the primary transplantation (3). Furthermore, it has been reported that splenectomy affects the concentration of serum immunoglobulins, in particular of the IgM-class (4, 5) and evokes a long-lasting lymphocytosis (6) predominantly consisting of B cells (7, 8). Furthermore, it has been found in previous studies on splenectomized, irradiated and fetal liver reconstituted mice, that the number of B lymphocytes in the bone marrow, lymph nodes, and Peyer's patches of these mice was significantly higher than in the same organs of sham-operated mice (8). These results were interpreted as an indication for the existence of a regulating compensatory mechanism at the B lymphocyte level in splenectomized mice. Nevertheless in these mice also a significant lower concentration of serum IgM was found (8). Besides these phenomena caused by adult splenectomy, neonatal splenectomy and congenital absence of the spleen in mice have been claimed to affect the ability of bone marrow and thymus cells to cooperate in an adoptive immune response against sheep red blood cells (SRBC) (9, 10).

In the present paper an experimental approach to locate the defect in the immune system responsible for the consistently low IgM concentration in the blood after splenectomy shall be described. Furthermore, the synergistic capacity of bone marrow and thymus cells of neonatally splenectomized mice in an adoptive plaque-forming cell (PFC) response against SRBC shall be analyzed.

MATERIALS AND METHODS

Animals. (C57BL/Rij x CBA/Rij) F1 female mice were used. They were purchased from the Medical Biological Laboratory, Rijswijk, The Netherlands and from The Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands.

Splenectomy. Adult splenectomy and sham-splenectomy were performed on mice at 14 weeks of age as described previously (11). Mice were anaesthetized with Nembutal (Abbott S.A., Saint-Remy-sur-Avre, France) (70 mg/kg body weight). There was no post-operative mortality.

Neonatal splenectomy and sham-splenectomy were performed on newborn mice within 24 hr after birth using the technique of Haller (12). Mice were anaesthetized by placing them on ice (0°C). If bleedings occurred during the splenectomy procedure, such mice were excluded from experiments, since bleeding resulted in spleen-nodules with a normal splenic histology (Rozing, unpublished observation). The abdomen of all splenectomized mice was carefully inspected for splenic remnants. There was less than 10% post-operative mortality.

Cell suspensions. Cell suspensions of spleen, bone marrow, and lymph nodes

(inguinal, brachial, axillary and mesenteric) were prepared in a balanced salt solution as described previously (13). Nucleated cells were counted with a Coulter Counter Model B. Results obtained from the femoral marrow were extrapolated to the marrow present in the whole animal using the data of Chervenick et al (14), who showed that in mice one femur contains 5.9 per cent of the total bone marrow. Corticosteroid resistant thymocytes (CRT) were obtained from 6-wk-old mice injected i.p. with 30 mg of the synthetic corticosteroid Dexamethasone sodium phosphate (Merck & Co., Rahway, New Jersey) per kg body weight 2 days previously.

Immunofluorescence staining of B cells. Before reacting with the conjugate, the cells were washed three times in a solution consisting of 5% bovine albumin in phosphate-buffered saline (5% BA-PBS). For detection of B lymphocytes a rhodamine conjugated goat anti-mouse immunoglobulin serum (TRITC-GaM-Ig) (Nordic, Tilburg, The Netherlands) was used in a direct vital staining technique. Details of the staining technique and the specificity of the TRITC-GaM-Ig have been described elsewhere (15). For detection of IgM-carrying B lymphocytes an identical procedure was used with a fluorescein conjugated specific rabbit anti-mouse-IgM serum (FITC-RaM-IgM). The FITC-RaM-IgM was a gift of Dr. J.J. Haaijman from the Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands. The TRITC-GaM-Ig reacted with all classes of mouse immunoglobulins, whereas the FITC-RaM-IgM reacted only with IgM.

Slides were examined with a Zeiss fluorescence microscope equipped with a vertical illuminator IV/F and an Osram HBO 50 mercury lamp. Cells with the morphology of lymphocytes displaying a ring, a cap or speckled peripheral fluorescence were scored as positive. Dead cells, which occurred occasionally, showing an uniform fluorescence were not scored.

X-irradiation. Recipient mice received 925 rad whole body X-irradiation, generated in a Philips Mueller MG 300 X-ray machine, as described in detail previously (15). Irradiated control mice died in 9-13 days.

Adoptive PFC response. Irradiated recipients were i.v. injected with the appropriate cell suspension and 4 x 10^8 SRBC within 4 hr after irradiation. Each group consisted of 5 mice. On the fourth day all mice were boosted with 4 x 10^8 SRBC i.p. The spleen of the recipients was removed on day 7 and the number of direct (IgM) plaqueforming cells (PFC) and indirect (IgG) PFC was determined in the plaque assay as described in detail elsewhere (13). Calculation of the 95% confidence limits associated with the number of plaques counted was done as described in a previous paper (13). The presence of IgG-PFC was accepted as significant when there was no overlap between the upper limit in the direct assay and the lower limit in the indirect assay.

Immunofluorescence staining of plasmacells. After washing the cells three times in 5% BA-PBS, cytocentrifuge slides were made using a Shandon Elliott Cytocentrifuge. Before reacting with the conjugate the cells were fixed for 20 min in cold ethanol-acetic acid (–20°C) according to the procedure as described by Vossen (16). For detection of Ig-containing and IgM-containing plasmacells the same antisera were

used as mentioned under B cell staining. Plasmacells were stained and quantitated as described by Vossen (16).

Serum immunoglobulins. Serum immunoglobulin concentrations (IgM, IgG_1, IgG_{2a}, IgG_{2b}, IgA) were determined using radial immunodiffusion plates commercially obtained from Meloy Laboratories, Inc., Springfield, Virginia.

RESULTS

Immunoglobulin positive (B) lymphocytes. The number of B lymphocytes was determined in the bone marrow and lymph nodes of both adult and neonatally splenectomized mice and in the spleen, bone marrow and lymph nodes of sham-operated animals 3-4 months after surgery. To estimate the total number of B cells we used a membrane-fluorescence technique with a specific anti-immunoglobulin conjugate as reagent, while a specific anti-IgM conjugate was used for the determination of IgM-positive B cells.

By comparing the number of B cells in the various organs of splenectomized and sham-splenectomized mice we found that in the bone marrow and lymph nodes of splenectomized mice the number of B lymphocytes was significantly higher (Table I). The increased number of B lymphocytes in splenectomized mice was caused by a specific enlargement of the B cell population in the various organs and not by a non-specific growth of the total cellularity of these organs, as can be concluded from the increased percentages of B cells. Also a significantly higher percentage of IgM-positive B lymphocytes was found in the bone marrow and lymph

TABLE I

Number of Ig-positive (B) Lymphocytes in Various Organs of Neonatally and Adult Splenectomized or Sham-Splenectomized Mice

Organ		ShSx[a]	Sx[a]	nShSx[b]	nSx[b]
spleen	%[c]	43.0 ± 2.9[e]	–	47.8 ± 2.5	–
	number[d]	6.9 ± 0.4	–	7.5 ± 0.4	–
bone marrow	%	9.8 ± 1.2	15.5 ± 0.7	10.7 ± 2.0	16.1 ± 0.4
	number	4.7 ± 0.6	6.8 ± 0.3	4.6 ± 0.9	8.0 ± 0.2
lymph nodes	%	23.1 ± 1.2	34.1 ± 2.8	23.5 ± 1.9	31.2 ± 2.2
	number	0.3 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.7 ± 0.1

[a] Mice were splenectomized (Sx) or sham-splenectomized (ShSx) between 14-20 weeks of age.
[b] Mice were neonatally splenectomized (nSx) or sham-splenectomized (nShSx) within 24 hr after birth.
[c] Percentage of B cells is expressed as percentage of all nucleated cells.
[d] Number of B cells (x 10^{-7}) is obtained by combining total cell numbers (data not shown) and percentages B cells as expressed under c.
[e] Average ± 1 S.E.M. Each group consisted of at least 5 mice.

nodes of splenectomized mice than in the same organs of sham-splenectomized mice (Table II). From the fact that the ratio between IgM-B cells and total B cells in the various organs is not influenced by splenectomy (Table II), it can be concluded that the predominancy of IgM carrying B lymphocytes remains after splenectomy and that the increase of the total number of B lymphocytes and the number of IgM-B lymphocytes is of about the same magnitude in the various organs.

TABLE II

Number of IgM-Positive (B) Lymphocytes in Various Organs of Neonatally and Adult Splenectomized or Sham-Splenectomized Mice

Organ			ShSx[a]	Sx[a]	nShSx[b]	nSx[b]
spleen	%	IgM-B cells[c]	35.0 ± 2.7[e]	–	39.4 ± 1.3	–
	ratio	IgM-B cells[d] / Ig -B cells	0.81 ± 0.01	–	0.83 ± 0.02	–
bone marrow	%	IgM-B cells	9.0 ± 0.9	14.7 ± 0.8	9.8 ± 1.6	14.8 ± 0.6
	ratio	IgM-B cells / Ig -B cells	0.92 ± 0.03	0.95 ± 0.03	0.93 ± 0.03	0.92 ± 0.02
lymph nodes	%	IgM-B cells	19.9 ± 1.7	29.5 ± 2.0	19.2 ± 1.5	26.1 ± 2.0
	ratio	IgM-B cells / Ig -B cells	0.86 ± 0.03	0.87 ± 0.02	0.82 ± 0.03	0.83 ± 0.01

[a] Mice were splenectomized (Sx) or sham-splenectomized (ShSx) between 14-20 weeks of age.
[b] Mice were neonatally splenectomized (nSx) or sham-splenectomized (nShSx) within 24 hr after birth.
[c] Percentage IgM-positive B cells is expressed as percentage of all nucleated cells.
[d] Ratio between IgM-positive B cells and all B cells is obtained by dividing the number of IgM-B cells by the total number of B cells.
[e] Average ± 1 S.E.M. Each group consisted of at least 5 mice.

Adoptive PFC response to SRBC. To test the ability of B lymphocytes of splenectomized mice to differentiate into antibody forming cells, 2 x 10^7 bone marrow cells of these mice were transferred into irradiated recipients in combination with 10^7 CRT and 4 x 10^8 SRBC. The CRT were added to avoid a limitation of the immune response by a lack of helper T cells. A dose of 10^7 CRT has been shown to be an optimal dose of helper T cells in this system (17). Using the cell transfer system, we found that in both groups of splenectomized mice the B lymphocytes in the bone marrow were fully capable to differentiate into IgM- and IgG-producing cells. Bone marrow cells from splenectomized mice gave rise to about twice as many IgM-PFC and IgG-PFC upon adoptive transfer into irradiated recipients, than did the same number of bone marrow cells from sham-splenectomized mice (Table III). This difference is of about the same magnitude as found for the total number of B cells (Table I) and the number of IgM-B cells (Table II), comparing the bone marrow of splenectomized and sham-splenectomized mice.

TABLE III

Adoptive PFC Response to SRBC of Bone Marrow Cells from Neonatally and Adult Splenectomized or Sham-Splenectomized Mice

Experimental group[a]	IgM-PFC/spleen[b]	IgG-PFC/spleen[b]
Sx[c]	17,410[e] (14,117 – 21,470)	59,866 (42,906 – 83,530)
ShSx[c]	8,397 (7,466 – 9,443)	25,466 (16,844 – 38,500)
nSx[d]	32,469 (28,002 – 37,648)	55,198 (40,845 – 74,593)
nShSx[d]	14,678 (13,520 – 15,935)	30,680 (19,808 – 47,520)

[a] 2 x 10^7 Bone marrow cells were transferred into irradiated recipients in combination with 10^7 CRT and 4 x 10^8 SRBC.
[b] Direct (IgM) and indirect (IgG) PFC assay was done on the spleen of the recipient mice 7 days after cell transfer.
[c] Mice were splenectomized (Sx) or sham-splenectomized (ShSx) between 14-20 weeks of age.
[d] Mice were neonatally splenectomized (nSx) or sham-splenectomized (nShSx) within 24 hr after birth.
[e] Geometric mean and 95% confidence limits. Each group consisted of at least 5 mice.

Immunoglobulin containing (C-Ig) cells. Immunoglobulin containing (C-Ig) cells were determined in bone marrow and lymph nodes of splenectomized mice and in spleen, bone marrow and lymph nodes of sham-splenectomized mice. As shown in Table IV the majority of plasmacells in sham-operated mice was located in the spleen and in the bone marrow, whereas a minor part of the plasmacells occurred

TABLE IV

Number of Ig-Containing Cells in Various Organs of Adult Splenectomized or Sham-Splenectomized Mice

Organ	Experimental group[a]	Total C-Ig[b] Cells x 10^{-3}	% C-IgM Cells[c]	Total C-IgM[b] Cells x 10^{-3}
spleen	ShSx	310 ± 35[d]	77 ± 6	239 ± 30
	Sx	–	–	–
bone marrow	ShSx	313 ± 48	22 ± 8	70 ± 23
	Sx	457 ± 62	30 ± 5	135 ± 28
lymph nodes	ShSx	38 ± 12	8 ± 4	3 ± 2
	Sx	63 ± 18	11 ± 7	7 ± 6
total[e]	ShSx	661		312
	Sx	520		142

[a] Mice were splenectomized (Sx) or sham-splenectomized (ShSx) between 14-20 weeks of age.
[b] Numbers of Ig containing cells (C-Ig) and IgM containing cells (C-IgM) are expressed per organ.
[c] Percentage IgM containing cells (C-IgM) is expressed as a percentage of all Ig containing cells (C-Ig).
[d] Average ± 1 S.E.M. Each group consisted of at least 6 mice.
[e] Total number of C-Ig cells and C-IgM cells summed over the three indicated organs.

in the lymph nodes. Regarding the distribution of IgM-containing (C-IgM) cells in these mice it is obvious that these cells were mainly located in the spleen. After splenectomy a higher number of C-Ig cells could be demonstrated in the bone marrow and lymph nodes of splenectomized mice as compared to the sham-operated animals. Also the number of C-IgM cells was higher in the organs of these mice. However, whereas the higher number of C-Ig cells in bone marrow and lymph nodes of splenectomized mice led to almost normal total numbers of C-Ig cells, the total number of C-IgM cells was only 50% of the number of C-IgM cells in sham-splenectomized mice.

Serum immunoglobulin concentration. In order to investigate the influence of splenectomy on the serum-immunoglobulin levels the concentrations of IgM, IgG_1, IgG_{2a}, IgG_{2b} and IgA were determined in the blood of neonatally and adult splenectomized and sham-splenectomized mice. No significant differences were found between splenectomized and sham-splenectomized mice for IgG_1, IgG_{2a}, IgG_{2b}, and IgA (Table V). The only clear effect of both adult and neonatal splenectomy on serum immunoglobulin concentrations occurred in the IgM class. As has been found in humans (4, 18), splenectomy of mice caused a significant decrease of the IgM concentration in the blood. Total serum immunoglobulin concentrations proved to be the same in splenectomized and sham-splenectomized mice.

TABLE V

Immunoglobulin Concentrations in the Blood of Neonatally and Adult Splenectomized or Sham-Splenectomized Mice

Experimental group	Immunoglobulin class					
	IgG_1	IgG_{2a}	IgG_{2b}	IgA	IgM	Total[a]
Sx[b]	2.13 ± 0.44[d]	1.76 ± 0.11	1.18 ± 0.05	1.37 ± 0.16	0.32 ± 0.03	6.76 ± 0.56
ShSx[b]	1.28 ± 0.13	1.86 ± 0.11	1.11 ± 0.07	0.91 ± 0.14	0.56 ± 0.03	5.72 ± 0.32
nSx[c]	2.08 ± 0.34	2.10 ± 0.18	1.11 ± 0.13	2.71 ± 0.46	0.29 ± 0.04	8.29 ± 0.92
nShSx[c]	2.24 ± 0.35	1.60 ± 0.07	0.87 ± 0.07	2.11 ± 0.54	0.60 ± 0.07	7.42 ± 0.67

[a] Total immunoglobulin concentration is obtained by summing the data of the various classes.
[b] Mice were splenectomized (Sx) or sham-splenectomized (ShSx) between 14-20 weeks of age.
[c] Mice were neonatally splenectomized (nSx) or sham-splenectomized (nShSx) within 24 hr after birth.
[d] Immunoglobulin concentrations (± 1 S.E.M.) in mg protein per ml. Each group consisted of at least 10 mice.

Bone marrow-thymus synergism. Neonatal splenectomy and congenital absence of the spleen have been reported to lead to a decreased capacity of bone marrow and thymus cells to cooperate in the adoptive PFC response to SRBC (9, 10). However, in previous experiments in our laboratory with splenectomized, irradiated and fetal liver reconstituted mice (8) no such failure could be demonstrated for bone

marrow and thymus cells of these mice. Since there is an analogy between the circumstances under which lymphocytes develop in neonatally splenectomized mice and in splenectomized irradiated and reconstituted mice, we investigated in the present experiments the synergistic ability of bone marrow and thymus cells of neonatally splenectomized mice in an adoptive PFC response to SRBC. Table VI shows that bone marrow cells of splenectomized mice transferred in combination with thymocytes from the same mice evoked an equal increase of the number of

TABLE VI

Ability of Bone Marrow Cells and Thymocytes of Neonatally Splenectomized or Sham-Splenectomized Mice to Cooperate in the Adoptive PFC Response to SRBC.

Experimental group[a]	IgM-PFC/spleen[b]	IgG-PFC/spleen[b]
BM^{nSX}	285[c] (154 – 432)	85 (n.s.)[d]
BM^{nSx} + TH^{nSx}	2,084 (1,673 – 2,596)	718 (n.s.)
BM^{nSx} + TH^{nShSx}	2,539 (1,914 – 3,368)	195 (n.s.)
BM^{nShSx}	736 (578 – 937)	400 (n.s.)
BM^{nShSx} + TH^{nSx}	7,707 (5,900 – 10,067)	5,771 (3,164 – 10,525)
BM^{nShSx} + TH^{nShSx}	10,489 (8,725 – 12,610)	319 (n.s.)

[a] 2 x 10^7 Bone marrow cells (BM) together with or without 2 x 10^7 thymocytes (TH) of mice splenectomized (nSx) or sham-splene[illegible]mized (nShSx) within 24 after birth were transferred into irradiated recipients together with . x 10^8 SRBC.

[b] Direct (IgM) and indirect (IgG) PFC-assay was done on the spleen of the recipient mice 7 days after cell transfer.

[c] Geometric mean and 95% confidence limits. Each group consisted of at least 5 mice.

[d] n.s. means that the number of PFC found in the indirect PFC-assay did not differ significantly from the number of PFC found in the direct PFC-assay.

PFC against SRBC as did these marrow cells transferred with thymocytes from sham-splenectomized mice, as compared to the transfer of such marrow cells alone. The same pattern occurred upon the transfer of bone marrow cells of sham-splenectomized mice in combination with or without thymocytes from splenectomized and sham-splenectomized mice. Since bone marrow cells of neonatally splenectomized mice, which were transferred together with cortison resistant thymocytes as helper T cells and SRBC, resulted in an excellent adoptive PFC response to SRBC (Table III), it can be concluded from these experiments that bone marrow and thymus cells of neonatally splenectomized mice are fully capable to cooperate in the immune response to SRBC. The differences in adoptive PFC responses to SRBC after transfer of bone marrow cells from either neonatally splenectomized or sham-splenectomized mice together with thymocytes and SRBC (Table VI) were probably the result of the non-optimal testsystem with normal thymocytes as helper T cells (17).

DISCUSSION

The spleen seems to be an important organ for the differentiation and maturation of hemopoietic stem cells to immunocompetent B lymphocytes. Although primarily the bone marrow is supposed to be involved in the first part of the B cell differentiation process, i.e. the continuous production of virgin B lymphocytes (19), there are a number of indications which suggest, that the first stage of the B cell differentiation can also occur in the spleen (15, 20). However, in normal mice probably the bone marrow is the major source of B lymphocytes (19), while the spleen is supposed to play an important role in the subsequent maturation of B cells (21). Basten et al. (22) were able to inactivate splenic B lymphocytes using a radioactively-labeled antigen, whereas bone marrow lymphocytes under identical conditions were unaffected. Furthermore, it was found by Stocker et al. (23) that per Ig-positive B lymphocyte the adoptive PFC response of spleen cells to the T cell-independent antigen dinitrophenylated polymer of flagellin (DNP-POL) is three times higher than the response of bone marrow cells. The difference in the length of time required to induce tolerance in bone marrow and splenic B cells, reported by Chiller and Weigle (24), may also be related to differences in antigen-binding capacities of the two cell populations, since the first step of tolerance induction is the binding of tolerogen to immunoglobulin receptors. Indeed Osmond and Nossal (25) found that the mean density of immunoglobulin receptors on splenic B lymphocytes is higher than on bone marrow B cells.

However, from the data presented in this paper and from experiments using splenectomized, irradiated and fetal liver reconstituted mice (8) it is obvious, that this maturation process can also occur outside the spleen. B lymphocytes in the bone marrow of splenectomized mice were found to be fully immunocompetent, since the increase in number of Ig-positive B cells (Table I) was related to a rise of the adoptive PFC response of bone marrow cells after transfer into irradiated recipients of the same magnitude (Table III). Obviously, in splenectomized mice not only compensation occurred for the absence of the splenic B cell population through an increase of the number of B lymphocytes in the remaining organs, but also the role of the spleen in the maturation process of B lymphocytes was taken over by the other organs. Since also the total number of immunoglobulin producing plasmacells in splenectomized mice was about the same as in sham-splenectomized mice (Table IV), corresponding with a normal serum immunoglobulin concentration in splenectomized mice (Table V), the compensatory mechanism probably regulating already on the B lymphocyte level, proved to result in an almost normal function of the humoral immune system.

Possibly the absence of a splenic factor with an "inhibitory effect" on B lymphocyte production in the bone marrow is responsible for the higher numbers of the various cell types of the B cell line in bone marrow and lymph nodes of splenectomized mice. Evidence has been presented by Dalos (26) and Maurice et al. (27) that irradiated spleens produce a factor which is inhibitory to bone marrow mitoses.

Probably such a factor is also produced by non-irradiated spleens, since Ernström and Sandberg (28) reported experiments in guinea pigs, in which these authors claimed the existence of a splenic factor inhibiting the production of lymphocytes in the thymus. On the other hand Auerbach (29) studying thymus lymphoid differentiation *in vitro* found a stimulation of thymic lymphopoiesis by embryonic spleen rudiments.

In contrast to total serum immunoglobulins, the IgM concentration in the blood of splenectomized mice was significantly reduced as compared to sham-operated mice (Table V). Such a decrease has also been found in patients after splenectomy (4, 18). The majority of IgM-synthetizing plasmacells in normal mice is located in the spleen (this study, 30). Obviously the low concentration of IgM in the blood of splenectomized mice was caused by a failure of the remaining organs to compensate completely for the absence of splenic IgM-producing plasmacells. Since in the bone marrow and lymph nodes the number of precursors of such plasmacells, the IgM-positive B lymphocytes, and their ability to differentiate into IgM-synthetizing PFC was not affected by splenectomy, probably the differentiation from IgM-positive B lymphocytes into IgM-producing plasmacells is not regulated optimally in these organs. Obviously the spleen provides a highly efficient environment for this differentiation step. Correspondingly patients splenectomized for various reasons show a failure to produce circulating IgM-antibodies upon intravenous injection of heterologous erythrocytes (31). Similar results were obtained in splenectomized rats and dogs by Van Wijck et al. (32) in the primary response after intravenous injection with sheep erythrocytes. Probably as a consequence of the reduced IgM-production, splenectomized individuals have a greater risk to develop fulminent and fatal sepsis due to normally non-fatal bacterial infections, such as pneumococcus and meningococcus infections (33). Also non-surgical asplenic states such as congenital absence of the spleen (34) and autosplenectomy in sickle-cell disease syndroms (35) display this increased risk of serious bacterial infection. In analogy with these data Shinefield and colleagues (36) reported that the susceptibility to infections with *Diplococcus pneumoniae* was increased in splenectomized mice. These bacteria do not have potent endotoxins (37) and the defense against these microorganisms greatly depends on the coating with opsonizing antibodies, which are predominantly of the IgM-class (38). The role of the spleen in the defense against such bacterial infections is a double one, i.e. the spleen provides besides a site of production of specific antibodies also a filter system over the blood stream, which permits phagocytosis of bacteria by the cells of the reticulo-endothelial system. Possibly the main effect of splenectomy resulting in the inability to prevent the development of fatal sepsis, is the loss of splenic phagocytic capacity. However, in an elegant set of experiments Haller (39) showed that in the defense against *D. pneumoniae* the production of opsonizing antibodies in the spleen was more important than the phagocytic capacity of this organ. This is in agreement with the findings of Benaceraff (40) who showed that the reticulo-endothelial activity in the liver is increased after splenectomy.

From *in vitro* studies on the interdependence of lymphoid organs during ontogeny

performed by Auerbach (29) it can be concluded that there exists during embryological life a stimulating splenic influence on the proliferation and differentiation of thymic cells. To study whether *in vivo* a splenic influence on B and/or T lymphocyte maturation in bone marrow and thymus exists during neonatal life, we analyzed the role of the spleen in the development of immune competence during that period. We examined the synergistic abilities of bone marrow and thymus cells of neonatally splenectomized mice. No negative effects of neonatal splenectomy were found on the functional capacities of either of these cell populations, suggesting that there is no *in vivo* regulatory influence of the spleen during neonatal life on lymphocyte differentiation and maturation. These results are in agreement with those reported by Auerbach and coworkers (41) in neonatally splenectomized mice and with our previous experiments about the cooperation between bone marrow and thymus cells of splenectomized, irradiated and reconstituted mice in the adoptive PFC response to SRBC (8). Nevertheless Bucsi et al. (9) and Wargon et al. (10) presented evidence, that bone marrow cells and thymocytes of congenital spleenless or neonatally splenectomized mice were defective in their capacity to cooperate. These contradictory observations are probably attributable to differences in sensitivity of the test system used, as suggested by Auerbach and colleagues (41).

ACKNOWLEDGEMENTS

We are very pleased to acknowledge Professor Dr. O. Vos for his valuable criticism during the performance of this study and Dr. J.J. Haaijman (Institute for Experimental Gerontology, Rijswijk, The Netherlands) for the generous gift of the FITC-conjugated rabbit anti-mouse-IgM serum. We also like to thank Mr. F. Luiten and Mr. A. van Oudenaren for technical assistance.

This investigation is part of a project program on the regulation of hemopoiesis, subsidized by the Netherlands Foundation for Medical Research (FUNGO). J. Rozing was supported by the Interuniversitary Institute for Radiation Pathology and Radiation Protection (Leiden, The Netherlands).

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Paper IV

B Lymphocyte Differentiation in Lethally Irradiated and Reconstituted Mice. I. The Effect of Strontium-89 Induced Bone Marrow Aplasia on the Recovery of the B Cell Compartment in the Spleen.

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B Lymphocyte Differentiation in Lethally Irradiated and Reconstituted Mice

I. The Effect of Strontium-89 Induced Bone Marrow Aplasia on the Recovery of the B Cell Compartment in the Spleen

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Received January 20, 1976

The influence of ^{89}Sr-treatment on the recovery of the B cell compartment in lethally irradiated, fetal liver reconstituted mice was studied by means of membrane fluorescence. ^{89}Sr is a bone-seeking radio-isotope which causes in a dose of 3 μCi ^{89}Sr/g body weight a depletion of all nucleated cells, including immunoglobulin-bearing (B) cells, of the bone marrow.

Treatment of irradiated and fetal liver reconstituted mice with 3 μCi ^{89}Sr/g body weight immediately and at 17 days after irradiation and reconstitution prevented recovery of the nucleated cell population, including B cells, in the bone marrow. In the spleen of such mice both nucleated cells and B cells reappeared at day 7 and 14 respectively. The B cell population in the spleen did not recover up to normal values during the experimental period of 45 days. It is concluded that B cell differentiation in lethally irradiated, fetal liver reconstituted mice can take place outside the bone marrow. The efficiency of this extra-medullary differentiation is discussed. The conclusion was drawn that mice with a ^{89}Sr-induced bone marrow aplasia are able to generate B lymphocytes. Consequently the bone marrow microenvironment seems not to be obligate to the differentiation of B lymphocytes. The peripheral lymphoid organs of such mice were found to be unable to compensate completely for the absence of B lymphocyte production in the bone marrow.

INTRODUCTION

Reappearance of immunological responsiveness after lethal irradiation and reconstitution with hemopoietic stem cells depends on the recovery of two types of lymphoid cells: B lymphocytes and T lymphocytes. By means of both membrane fluorescence studies (1) and functional tests (2, 3) it was shown that the B cell population recovers faster than the T cell population. Comparison of the contribution of various lymphoid organs to the recovery of the B cell population in lethally irradiated and fetal liver reconstituted mice, revealed that the B cell compartment of the spleen recovered somewhat earlier than the B cell populations in the other organs tested (1, 4, 5). Since in these studies B cells were assayed using surface-immunoglobulin as a marker, it is possible that immunoglobulin negative percursor B cells are produced outside the spleen, migrate towards the spleen and mature there into immunoglobulin positive cells, as has been hypothesized by Osmond and Nossal (6–8).

In normal mice the bone marrow is the major site of lymphocyte production (7, 9). These newly formed lymphocytes are probably immature stages of the B cell line (6, 10). Initially these cells are immunoglobulin negative. During maturation they acquire increasing amounts of surface immunoglobulins (7). This maturation process can take place in the bone marrow but also in peripheral lymphoid organs (7, 11). A continuous transport of newly formed lymphocytes from the bone marrow towards spleen and lymph nodes has been demonstrated in guinea pigs and rats (10, 12).

The present study deals with experiments which were performed to investigate the contribution of the bone marrow to the recovery of the B cell compartment in irradiated and fetal liver reconstituted mice. To prevent lymphocyte production and B cell maturation in the bone marrow we induced an experimental medullary aplasia by treating the mice with a radioactive strontium isotope, ^{89}Sr, which is readily incorporated in bones. The results suggest that the bone marrow is involved in the generation of the B cell population after irradiation and reconstitution. The contribution of the bone marrow, however, is not obligate to this recovery.

MATERIALS AND METHODS

Animals. (C57BL/Rij × CBA/Rij)F1 female mice, 10–12 weeks old, were used. They were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands.

X-irradiation. Recipient mice received 850 rad whole body X-irradiation generated in a Philips Müeller MG 300 X-ray machine. Animals were irradiated in aerated circular Perspex cages. Physical constants were: 300 kV; 10 mA; HVL 3 mmCu; dose rate of 60 rad/min; focus object distance 53 cm. Irradiation was corrected for field inhomogeneity. The cage was placed on a layer of 11 cm hardboard. This resulted in maximal backscatter. A Baldwin Ionex dosimeter was used to measure the dose during irradiation. Irradiated control mice died in 9–11 days.

Reconstitution. Lethally irradiated mice were reconstituted with 7.5×10^6 syngeneic fetal liver cells. These cells were injected intravenously (iv) within 2 hr after irradiation. Fetal livers were obtained from mice at 14 days of gestation. At that time virtually no immunoglobulin-bearing cells are present in fetal liver (less than 1 per 10^4 nucleated cells).

Strontium-89. Mice were injected intraperitoneally (ip) with either 1, 2, or 3 μCi ^{89}Sr per gram body weight. The mice were anesthesized with ether during this procedure. ^{89}Sr is a high-energy (1.47 Mev), β-particle emitter with a half-life time of 50.4 days. It is a bone-seeking isotope which makes the marrow cavity a hostile environment for cell proliferation. After iv or ip injection ^{89}Sr is incorporated in the bone within 2 hr (13, 14). In our experiments ^{89}Sr was injected ip since inoculation via this route results in 1.5 times more skeletal deposition of the isotope as compared with intravenous injection (15). The effect on the marrow cell population is dose-dependent (Fig. 1). As demonstrated by Nillson and other authors (14, 16, 17) ^{89}Sr is built-in rather equally throughout the skeleton. From both histological studies (17–19) and survival experiments using splenectomized recipients (20–22) it was established that only with fairly high doses ^{89}Sr, i.e., 3 μCi ^{89}Sr/g body weight or more, a complete bone marrow

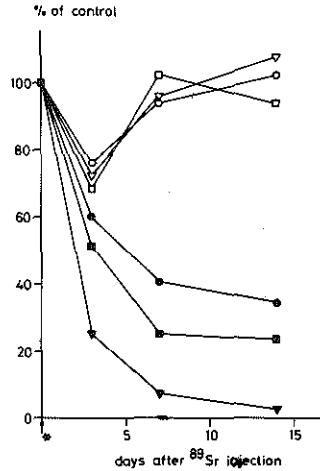

FIG. 1. The number of nucleated cells in spleen and bone marrow of normal mice ip injected (*) with either 1 μCi ^{89}Sr/g (circles), 2 μCi ^{89}Sr/g (squares), or 3 μCi ^{89}Sr/g (triangles). (○, □, ▽) Spleen and (●, ■, ▼) bone marrow. The number of nucleated cells found in an organ is expressed as the percentage of the number of nucleated cells in normal mice. Control mice received 0.5 ml 0.9% NaCl ip. The injection of NaCl did not influence the number of nucleated cells in spleen and bone marrow.

aplasia could be acquired in mice. ^{89}Sr was obtained from Amersham, England, as strontium chloride in aqueous solution, less than 0.6% contaminated with other isotopes. Control mice received an ip injection with an equal volume of 0.9% NaCl.

Whole-body counting. The retention of ^{89}Sr in mice was followed by applying whole-body counting (14). Mice were centrally located under a NaI (Th) scintillation detector which was connected with a gamma pulse-height analyzer.

Cell suspensions. Immediately after killing mice with ether, spleens and femurs were removed and brought into a balanced salt solution (BSS). This solution was prepared according to Mishell and Dutton (23) and supplemented with 5% newborn calf serum. Bone marrow was obtained by flushing the femurs with BSS. Spleens and bone marrow were minced with scissors and squeezed through a nylon-gauze filter to give a single cell suspension. Nucleated cells were counted with a Coulter Counter Model B.

Fetal livers. Mouse embryos were obtained by putting single breeding pairs to a cage. Time of conception was determined by inspecting the female mice for vaginal plugs every morning. At day 14 (day 0 represents the day after the night of conception) pregnant mice were selected and used as embryo donors. After killing the mice with ether, the uteri were removed and placed in BSS. Subsequently embryos were dissected from the uteri. The fetal livers were removed under a dissection microscope and pooled in BSS. Cell suspensions were prepared as described above.

Immunofluorescence staining of B cells. Before reacting with the antiserum, the cells were washed three times in phosphate-buffered saline supplemented with 5% bovine albumin (BA-PBS) (24). For the detection of B lymphocytes 25 μl aliquots of 10^6 cells were added to 25 μl of a rhodamine conjugated goat anti-mouse immunoglobulin serum (TRITC-GαM-Ig) (Nordic, Tilburg, The Netherlands)

and incubated at 4°C for 30 min with gentle shaking every 10 min. After the incubation, the cells were washed three times with an 1% BA-PBS solution and resuspended in approximately 30 μl. One drop of the cell suspension was mounted on a slide in an equal volume of buffered glycerol (9 parts glycerol, 1 part PBS) and covered with a coverslip. The edges were sealed with paraffin.

The TRITC-GαM-Ig reacted with all classes of mouse Ig as well as with Ig kappa and Ig lambda light chains. Specificity of the conjugate was tested using the Defined Antigen Substrate Spheres (DASS) system (25).

The slides were examined with a Zeiss fluorescence microscope equipped with a vertical illuminator IV/F and an Osram HBO 50 mercury lamp. Cells with the morphology of lymphocytes displaying a ring, a cap or speckled peripheral fluorescence were scored as positive. Dead cells showing an uniform fluorescence were not scored.

RESULTS

Effect of ^{89}Sr-treatment on bone marrow and spleen in normal mice. In order to find an appropriate dose of ^{89}Sr to obtain a complete bone marrow aplasia in mice normal non-irradiated animals were injected ip with various doses of ^{89}Sr. The doses used were 1 μCi, 2 μCi, and 3 μCi per gram body weight. At 3, 7, and 14 days after injection the number of nucleated cells and the number of B cells in spleen and bone marrow of the recipient mice were determined. The effect of the various doses ^{89}Sr on the total cellularity in spleen and bone marrow is shown in Fig. 1. After ^{89}Sr-injection a reduction of the number of nucleated cells in bone marrow was found in all groups of mice. After 14 days 34.5% of the normal number of bone marrow cells remained in the 1 μCi ^{89}Sr/g group, 24.0% in the 2 μCi ^{89}Sr/g group and only 2.8% in the 3 μCi ^{89}Sr/g group. After ^{89}Sr treatment also a drop in the total number of nucleated cells of the spleen could be observed. After three days this decrease was followed by a recovery of the number of nucleated cells up to normal values in all the groups tested.

Treating mice with ^{89}Sr resulted in a decrease of the number of B cells (Fig. 2). From the third day on the number of B cells in the spleen remained at a constant and rather dose-dependent level. The number of B cells in the bone marrow, however, continued to diminish. This resulted in a virtual absence of B cells in the bone marrow of mice treated with 3 μCi ^{89}Sr/g after 7 days. The bone marrow of the 2 μCi ^{89}Sr/g and the 1 μCi ^{89}Sr/g group contained 4.8% and 13.6% of normal values respectively at 14 days after injection. Since the dose of 3 μCi ^{89}Sr/g body weight completely depleted the bone marrow of nucleated cells, this dose was used in further experiments on the role of the bone marrow in the recovery of the B cell compartment in the spleen after irradiation and reconstitution with fetal liver cells.

In mice treated with the above mentioned doses of ^{89}Sr also the overall retention of ^{89}Sr was followed using whole-body counting. In all the groups tested a constant fall of the ^{89}Sr-activity was found (Table 1). This can be ascribed only in part to the relative short half-life time of ^{89}Sr (50.4 days). Probably also the continuous replacement of incorporated ^{89}Sr by calcium from the normal diet (26) contribute to this rapid fall of ^{89}Sr-activity. In all the groups tested a negative relationship could be demonstrated between the specific ^{89}Sr-activity as measured by whole-body counting and the number of nucleated cells in the bone marrow of individual

TABLE 1

The Retention of ^{89}Sr in Normal Mice as Measured by Whole-Body Counting

Days after[a] injection	Counts/min[b]		
	1 μCi $^{89}Sr/g$	2 μCi $^{89}Sr/g$	3 μCi $^{89}Sr/g$
3	8350	17,084	22,793
7	7459	15,205	22,454
14	6576	12,986	15,898

[a] Mice were ip injected with either 1, 2, or 3 μCi $^{89}Sr/g$ body weight respectively. At day 3, 7, and 14 after injection two mice of each group were killed and used for ^{89}Sr-measurement.

[b] The arithmatic mean of the determination in two mice is presented.

mice, showing r-values varying between -0.95 and -0.99. Therefore in further experiments whole-body counting was used as a control for technical failures (e.g., injection errors). This technique also allowed measurement of removal of ^{89}Sr from the body. A level of 16,000 counts/min (Table 1) was arbitrarily taken as the lowest permissible limit to maintain a complete depletion of the marrow.

Effect of ^{89}Sr-treatment on bone marrow and spleen in irradiated and reconstituted mice. To investigate the influence of ^{89}Sr-induced bone marrow aplasia on the recovery of the B cell compartment of mice after irradiation and reconstitution, one group of lethally irradiated and fetal liver reconstituted mice was injected ip with 3 μCi $^{89}Sr/g$ immediately after the reconstitution. The control group was injected ip with the same volume 0.9% NaCl. Since at 14 days after irradiation and reconstitution the whole-body count of the ^{89}Sr injected animals almost reached the level which had been taken as the lowest permissible limit (Table 2), the mice left received another ip injection of 3 μCi $^{89}Sr/g$ at day 17. This amount of ^{89}Sr proved to be sufficient throughout the experimental period.

At various intervals after irradiation and reconstitution the number of nucleated cells and B cells was determinated in spleen and bone marrow. Up to 7 days after irradiation an enormous decrease of the number of nucleated cells in bone marrow of both ^{89}Sr-treated mice and control mice was observed (Fig. 3). After this decrease a fast repopulation of the bone marrow of the control mice could be demonstrated, reaching normal values at day 28. Contrariwise the number of nu-

TABLE 2

The Retention of ^{89}Sr in Irradiated and Fetal Liver Reconstituted Mice as Measured by Whole-Body Counting

Days after injection[a]	Counts/min[b]
7	21,346 ± 662
14	16,518 ± 676
20	32,399 ± 3080
28	26,750 ± 2835
45	19,857 ± 1271

[a] At 17 days after the first ip injection of 3 μCi $^{89}Sr/g$ body weight all mice received another ip injection with the same amount of ^{89}Sr.

[b] Average ±1 SEM. At least five mice were used for one determination.

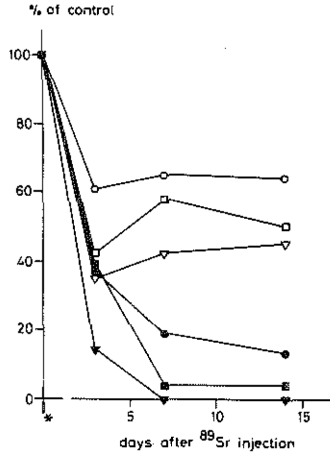

FIG. 2. The number of immunoglobulin-positive (B) cells in spleen and bone marrow of normal mice after ip injection (*) with either 1 μCi ^{89}Sr/g (circles), 2 μCi ^{89}Sr/g (squares), or 3 μCi ^{89}Sr/g (triangles) as determined by membrane fluorescence. (○, □, ▽). Spleen and (●, ■, ▼) bone marrow. The number of B cells found in an organ is expressed as the percentage of the number of B cells in that organ in normal mice. Control mice received 0.5 ml 0.9% NaCl ip The injection of NaCl did not influence the number of B cells in spleen and bone marrow.

cleated cells in the bone marrow of ^{89}Sr-treated mice remained at a level of 6.0% of the normal value throughout the experimental period. The number of B cells in the bone marrow of both groups of mice also decreased up to day 7 (Fig. 4). Thereafter a recovery of the B cell population in the bone marrow of the control mice was found reaching normal values at day 30. In the bone marrow of ^{89}Sr-treated mice B cells were scored only very occasionally.

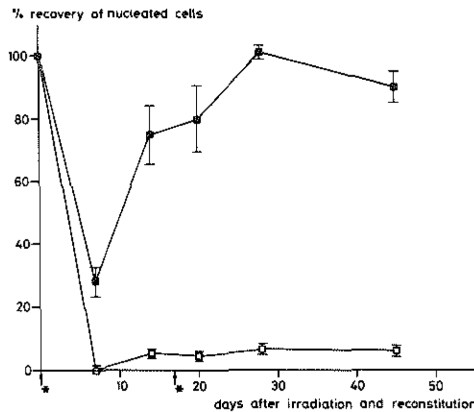

FIG. 3. The recovery of the number of nucleated cells in the bone marrow of lethally irradiated, fetal liver reconstituted mice treated with ^{89}Sr (□) or 0.9% NaCl (■). Mice treated with ^{89}Sr received 3 μCi ^{89}Sr/g body weight ip immediately after irradiation and reconstitution (*) and another ip injection with the same amount of ^{89}Sr 17 days (*) later. Control mice were inoculated with 0.9% NaCl at the same time. The numbers of nucleated bone marrow cells are expressed as percentages of the number of nucleated marrow cells of normal mice. Each figure represents the average ± 1 SEM. For each figure at least five mice were used.

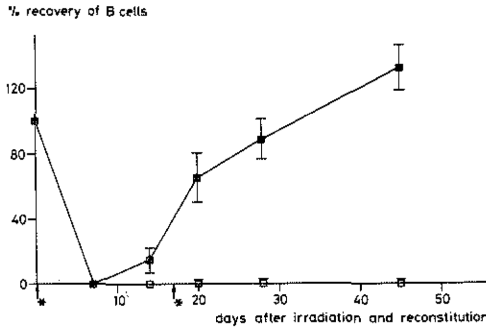

FIG. 4. The recovery of the number of immunoglobulin-positive (B) cells in the bone marrow of lethally irradiated, fetal liver reconstituted mice treated with ^{89}Sr (□) or 0.9% NaCl (■) as determined by means of membrane fluorescence. Mice treated with ^{89}Sr received 3 μCi ^{89}Sr/g body weight ip immediately after irradiation and reconstitution (*) and another ip injection with the same amount of ^{89}Sr 17 days (*) later. Control mice were inoculated with 0.9% NaCl at the same time. The numbers of B cells in bone marrow are expressed as percentages of the number of B cells in the bone marrow of normal mice. Each figure represents the average ± 1 SEM. For each figure at least five mice were used.

In the spleen of both ^{89}Sr-injected and control mice also a decrease in the number of nucleated cells was observed until 7 days after irradiation (Fig. 5). This decrease was followed by a continuous recovery of the cellularity of the spleen of control mice reaching the normal level at day 45. In the spleen of ^{89}Sr-injected mice, the number of nucleated cells recovered faster than in control mice, reaching nearly normal cell numbers already at day 14. After this peak at day 14 the cellularity of the spleen of ^{89}Sr-treated mice decreased, terminating at a level of 75% of normal values at day 20.

The influence of ^{89}Sr on the recovery of the B cell compartment in the spleen

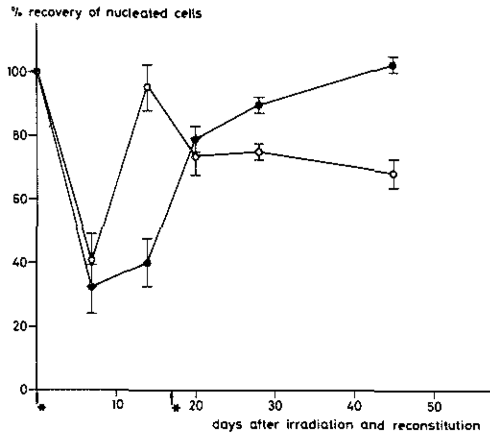

FIG 5. The recovery of the number of nucleated cells in the spleen of lethally irradiated, fetal liver reconstituted mice treated with ^{89}Sr (○) or 0.9% NaCl (●). Mice treated with ^{89}Sr received 3 μCi ^{89}Sr/g body weight ip immediately after irradiation and reconstitution (*) and another ip injection with the same amount of ^{89}Sr 17 days (*) later. Control mice were inoculated with 0.9% NaCl at the same time. The numbers of nucleated spleen cells are expressed as percentages of the number of nucleated spleen cells of normal mice. Each figure represents the average ± 1 SEM. For each figure at least five mice were used.

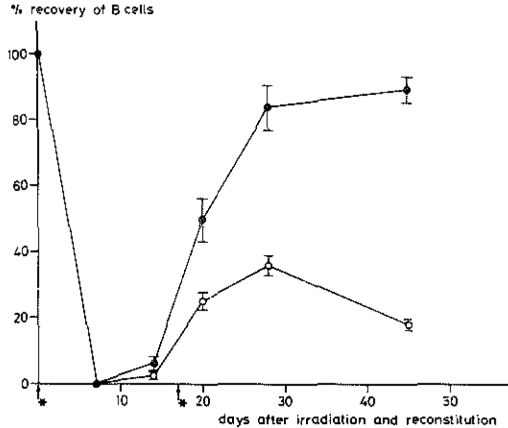

FIG. 6. The recovery of the number of immunoglobulin-positive (B) cells in the spleen of lethally irradiated, fetal liver reconstituted mice treated with ^{89}Sr (○) or 0.9% NaCl (●) as determined by means of membrane fluorescence. Mice treated with ^{89}Sr received 3 μCi ^{89}Sr/g body weight ip immediately after irradiation and reconstitution (*) and another ip injection with the same amount of ^{89}Sr 17 days (*) later. Control mice were inoculated with 0.9% NaCl at the same time. The numbers of splenic B cells are expressed as percentages of the number of B cells in the spleen of normal mice. Each figure represents the average ± 1 SEM. For each figure at least five mice were used.

is shown in Fig. 6. After the initial decrease in both ^{89}Sr-treated and control mice a steady recovery of the number of B cells in the spleen of control mice was found starting at day 14 and reaching almost normal levels at day 45. The recovery of the B cell population in the spleen of ^{89}Sr-treated mice also started at day 14. However, the rate of recovery of the B cell population in the spleen of these mice was smaller than in control mice. The B cell compartment in the spleen of ^{89}Sr-treated mice never reached normal or nearly normal cell numbers during the experimental period. At day 28 the number of B cells in the spleen of ^{89}Sr-treated mice reached the maximum level of 36% of the normal value.

DISCUSSION

The bone marrow of rodents represents a place of continuous and large-scale lymphocyte production (6, 7, 27–29). Yoshida and Osmond (30) demonstrated in guinea pigs that lymphopoiesis is maintained principally by proliferation of large lymphoid cells instead of a continuous differentiation from stem cells. Recently Osmond (8) suggested that stem cells, which are Ig negative (31), differentiate into large lymphoid cells within the bone marrow. These cells are also Ig negative, extensively self-replicating and the progenitors of marrow small lymphocytes (7, 29, 30, 32–34). Lymphocytes in bone marrow of normal mice demonstrate a large diversity in Ig density. Such a variation is not found in lymphocytes in spleen and lymph nodes (6, 35, 36). Approximately 30% of the small lymphocytes in the bone marrow show an Ig density comparable with Ig-bearing lymphocytes in spleen and lymph nodes. Twenty percent of the marrow lymphocytes bear less, but still demonstrable surface Ig. The other 50% of the small marrow lymphocytes show no detectable surface Ig nor do they bear the θ-antigen, which is a characteristic marker for T lymphocytes (37). Cells without surface

Ig or θ-antigen are also found in lymph nodes and spleen of adult mice, but in a much smaller proportion than in bone marrow (38). Using isotopic labelling techniques Osmond and Nossal (7) provided evidence that these double negative cells in bone marrow express increasing amounts of Ig during differentiation and proliferation. They concluded that these double negative cells are immature stages of the B cell line.

Basten, Miller, Warner, and Pye (11) using a "suicide" technique with radioactively-labelled antigen demonstrated that bone marrow lymphocytes are unaffected in conditions in which B lymphocytes in the spleen are inactivated. This might suggest that the maturation of B lymphocytes to antigen-binding cells takes place mainly after the cells have left the marrow.

The appearance of Ig positive cells has been studied in ontogeny (39, 40) and after irradiation and reconstitution with hemopoietic stem cells (1, 4, 5, 39). In these latter studies fetal liver cells were used as a source of stem cells. Ig positive cells were found to appear in the spleen before they could be demonstrated in the other lymphoid organs. The contribution of bone marrow to the recovery of the B cell population after irradiation and reconstitution can be prevented by treatment with ^{89}Sr (17, 22). From the results presented here it is obvious that remaining nucleated cells can still be found in the bone marrow of ^{89}Sr-treated mice. These cells are reported to be predominantly reticular cells, mononuclear phagocytes and granuloid cells (14, 17, 19). Since one of these cell types may provide a microenvironment for B cell differentiation, it cannot be excluded completely that recovery of the B cell population found in other organs than marrow in ^{89}Sr-treated animals depends in part on a marrow microenvironment-dependent differentiation step. Further maturation and proliferation could then occur at extramedullary sites. However, cells probably responsible for inductive influences on differentiation seem to be rather radiosensitive functionally, although they are morphologically radio-resistant (41–43). Furthermore, since microenvironmental influences are supposed to work only on a very close distance like cell-to-cell contact or a humoral factor in a very low concentration (44) it seems to be improbable that cells present in the marrow after ^{89}Sr-treatment have a regulatory influence upon B cell differentiation in other lymphoid organs.

Philips and Miller (45) using ^{89}Sr-treated irradiated and bone marrow reconstituted mice have shown that such mice can evoke a PFC response in the spleen. They concluded that B cell differentiation can occur without lymphopoiesis in the bone marrow. The data presented in this paper about the influence of ^{89}Sr-treatment of the recipient mice upon the appearance of B cells in the spleen are in agreement with their results. In the present experiments total B cell count in the spleen by means of membrane fluorescence allowed a quantitative study of the influence of excluding the production of cells in the bone marrow. The number of B cells in the spleen of ^{89}Sr-treated mice raised up to only 36% of the normal value during the experimental period. Klassen and coworkers (21) demonstrated that the spleen of ^{89}Sr-treated mice receives only a minimal dose of radiation from the injected ^{89}Sr. Therefore it is unlikely that the irradiation dose is a main factor in the decrease of the number of B cells in the spleen with over 60%. However there may be several other explanations for this phenomenon:

(1) In normal irradiated and reconstituted mice there probably is a continuous migration to the spleen of bone marrow-derived lymphoid cells of the B cell line.

This hypothetical transport is absent in ^{89}Sr-treated irradiated and reconstituted mice;

(2) Hemopoiesis in ^{89}Sr-treated animals is only present in the spleen (20, 21, 46). Therefore competition for stem cells may occur between the various cell lines generated in this organ. Regarding the stem cell compartment in ^{89}Sr-treated mice Fried, Gurney and Swatek (47) demonstrated a persistent increased number of CFU-S in the spleen of those mice as compared with control mice. In spite of this increase of splenic CFU-S the total number of CFU-S in ^{89}Sr-treated mice appeared to be much smaller than in normal mice. Therefore the stem cell pool may be quantitatively a limiting factor in the generation of B cells in ^{89}Sr-treated mice;

(3) Although virgin B cells are reported to be non-recirculating cells (48) it cannot be excluded that some B cells are eliminated while circulating in the blood through the marrow cavity.

Since termination of these experiments a paper appeared by Kincade, Moore, Schlegel and Pye (49) describing B cell development in a similar system. Although they contented themselves to inject a single dose of 100 μCi ^{89}Sr iv their results are compatible with those presented here.

From the results presented here and from those of Kincade *et al.* (49) it can be concluded that mice with a ^{89}Sr-induced bone marrow aplasia are able to generate B lymphocytes. Consequently the bone marrow microenvironment seems not to be obligate to the differentiation of B lymphocytes. The peripheral lymphoid organs of such mice were found to be unable to compensate completely for the absence of B lymphocyte production in the bone marrow.

ACKNOWLEDGMENTS

We are very indebted to Professor Dr. O. Vos, Dr. W. Hijmans (Institute for Experimental Gerontology, Rijswijk, The Netherlands), and Dr. O. B. Zaalberg (The Medical Biological Laboratory, Rijswijk, The Netherlands) for their valuable criticism during the preparation of this manuscript. The experiments with ^{89}Sr were performed in the Radiobiological Institute (Rijswijk, The Netherlands) under supervision of Professor Dr. L. M. van Putten, to whom the authors are very grateful for the facilities provided. Thanks are also expressed to Mrs. J. de Ruiter for excellent technical assistance and to Dr. J. Bloemmen (Institute of Experimental Gerontology, Rijswijk, The Netherlands) for determining the specificity of the goat anti-mouse immunoglobulin conjugate.

This investigation was supported by the Interuniversitary Institute of Radiationpathology and Radiation Protection (Leiden, The Netherlands).

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Paper V

B Lymphocyte Differentiation in Lethally Irradiated and Reconstituted Mice. IV. A Histological Study Using Immunofluorescent Detection of B Lymphocytes in Sections of Spleen, Lymph Nodes and Peyer's Patches.

J. Rozing, N.H.C. Brons, W. van Ewijk and R. Benner.

B Lymphocyte Differentiation in Lethally Irradiated and Reconstituted Mice

IV. A Histological Study Using Immunofluorescent Detection of B Lymphocytes in Sections of Spleen, Lymph Nodes and Peyer's Patches

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The recovery of B lymphocytes was investigated in mice after irradiation and reconstitution with fetal liver cells. B cell recovery was studied specifically with immunoflorescence on frozen sections of spleen, lymph nodes and Peyer's patches. B lymphocytes were observed in the spleen at day 8, a few days before they could be detected in lymph nodes and Peyer's patches (day 13). The first B cells in the spleen were found in the central part of the periarteriolar lymphatic sheath (PALS). Later on, the number of B lymphocytes increased in the spleen and B cells formed growing follicles at the periphery of the PALS. Subsequently, brightly fluorescent B cells appeared in the marginal zone, which surrounded the follicles. Possibly these strongly fluorescent B cells in the marginal zone represent a distinct subpopulation of B lymphocytes, i.e. B memory cells. Another two weeks later, around day 30, also germinal center formation was observed in the follicles of the spleen.

B cell development in lymph nodes and Peyer's patches started somewhat later than in the spleen, but once started, structural recovery of the B cell compartment was completed very fast in these organs. It is suggested, that B cell recovery in the spleen was caused by an influx of immature B lymphocytes from the bone marrow, whereas more mature B lymphocytes were involved in the B cell recovery in lymph nodes and Peyer's patches. These mature B cells were possibly derived from the spleen.

Germinal center reactions in lymph nodes and Peyer's patches were found at day 25 and occurred before the formation of germinal centers in the spleen, but after the appearance of strongly fluorescent cells in the marginal zone of the spleen. Apparently, germinal center formation is not essential for the recovery of the population of very brightly fluorescent B cells in the marginal zone after irradiation and reconstitution.

INTRODUCTION

In rodents B lymphocytes and T lymphocytes occupy different areas in peripheral lymphoid organs. In the spleen most lymphocytes are located in the white pulp, which is organized around branches of the splenic artery and consists of the periarteriolar lymphatic sheath (PALS) and the follicles. B cells occur mainly in the peripheral PALS and the surrounding follicles (1, 2), while T lymphocytes are present predominantly in the central area of the PALS (3). In lymph nodes B cells occupy also follicles, and non-follicular areas localized in the outer cortex, whereas T lymphocytes are situated in the paracortex (4).

These structures, however, are no statical constructions. The various lymphoid cell types are probably continually migrating, recirculating or reacting upon anti-

genic stimulation. In the spleen, recirculation occurs through the blood stream and the lymphatics. B and T lymphocytes enter the white pulp both through the marginal zones which separate the white pulp from the surrounding red pulp (5, 6). Upon entering the white pulp, B and T lymphocytes segregate each in distinct compartments (7, 8). Lymphocytes entering the white pulp later escape through marginal zone bridging channels, and enter red pulp sinuses (9). Via these sinuses the cells are transported to the venous circulation.

In lymph nodes and Peyer's patches postcapillary venules are the most important site of entry for both B and T lymphocytes (10, 11), whereas a minority of these cells enter the lymph nodes through the afferent lymphatics (12). The major route for lymphocytes to leave the lymph nodes is by the efferent lymphatics (10, 11). Besides the interactions between the lymphoid cells and their environments also the formation of new lymphocytes and the mixing of these cells with those already present continuously occurs.

In normal mice the bone marrow probably is the major source of B lymphocytes (13, 14). However, these experiments do not give much information about the interrelationship between the differentiation of B lymphocytes in the bone marrow and B cell compartments elsewhere. From studies of Brahim and Osmond (15), using a selective bone marrow labeling technique with ^{3}H-thymidine *in vivo*, it can be concluded, that a continuous transport of newly formed lymphocytes from the bone marrow towards spleen and lymph nodes through the blood stream indeed takes place.

In previous investigations in our laboratory on B cell differentiation in lethally irradiated and reconstituted mice (16), we obtained evidence that under these experimental circumstances the bone marrow also plays an important role in the recovery of the B cell population in these animals. This model provides the opportunity to study the events concerning new formation of B lymphocytes without any interference of existing B cells. Besides the bone marrow as an important site for B lymphocyte production in irradiated and reconstituted mice, the spleen of these animals may be of equal importance for the maturation and differentiation of B lymphocytes (17, 18). However, since with respect to early B cell differentiation no essential role of any lymphoid organ could be established in irradiated and reconstituted mice as well as in normal animals (16, 19-21) another explanation for this apparently multifocal phenomenon has to be considered. Nieuwenhuis and Keuning (22) postulated that a common structure in lymphoid organs, like the germinal centers in the follicular systems in spleen, lymph nodes and gut-associated lymphoid tissue are involved in the generation of B lymphocytes.

To investigate whether these structures in the various lymphoid organs are involved in B cell differentiation after irradiation and reconstitution, and to determine the possible migration pathways of newly differentiated B lymphocytes, we examined in the present experiments various lymphoid tissues of irradiated and reconstituted mice histologically with an immunofluorescence technique. Furthermore the recovery pattern of the various B cell compartments, i.e. primary follicles, marginal zone B lymphocytes and germinal centers, was studied and a possible relationship

between the appearance of B lymphocytes in the marginal zone on one side and the occurrence of follicular systems on the other side, will be discussed.

MATERIALS AND METHODS

Animals. (C57BL/Rij X CBA/Rij) F1 female mice, 10-14 weeks old, were used. They were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands.

X-irradiation and reconstitution. Recipient mice received 925 rad whole body X-irradiation generated in a Philips Mueller MG 300 X-ray machine, as described in detail previously (16). Irradiated control mice died in 9-13 days.

Lethally irradiated mice were reconstituted with 1.5 x 10^6 syngeneic fetal liver cells. These cells were injected intravenously (i.v.) within 2 hours after irradiation. Fetal livers were obtained from mice at 14 days of gestation. At that time virtually no immunoglobulin-bearing cells are present in fetal liver (less than 1 per 10^4 nucleated cells).

Preparation of tissue. At various times after irradiation and reconstitution groups of 5 mice were sacrificed and used to investigate the recovery of the B cell population in spleen, lymph nodes (mesenteric, inguinal, axillary, and brachial) and Peyer's patches. Since removal of the peripheral blood from arterioles, sinuses and venules in the various lymphoid organs greatly facilitates light microscopical investigation of the structure of these organs, the mice received a total body perfusion with oxygenated phosphate buffered saline (PBS) containing 0.1 per cent procaine and 0.2 per cent dextran –40, pH 7.2; 300 milliosmols. Full technical details of this procedure have been described by Van Ewijk et al. (3). Animals were anaesthetized with Nembutal (70 mg/kg body weight). Perfusion was stopped after bleaching of the liver. Immediately thereafter spleen, lymph nodes and Peyer's patches were excised.

To study the appearance of Ig-positive (B) cells in spleen, lymph nodes and Peyer's patches we employed the immunofluorescence technique described by Gutman and Weissman (4). Fresh slices of lymphoid tissues perfused with PBS were impregnated in Tissue-Tek II (Division Miles Laboratories, Naperville, Illinois, U.S.A.) and mounted in gelatin capsules (Eli Lilly and Company, Indianapolis, U.S.A., nr. 00). The capsules were then frozen in solid carbon dioxide and stored at –70°C. Prior to sectioning the gelatin capsule was removed with a razor blade. The frozen specimen was mounted on a specimen stage with fresh Tissue-Tek and cut with a Bright 5030 rocking microtome (Bright Instrument Company Ltd., Huntingdon, England), at –25°C. Serial 5μ thick sections were collected on thoroughly cleaned microscope slides. The sections were fixed immediately by dipping the slides less than one second in distilled acetone. The air dried sections were stored at 4°C.

To visualize B lymphocytes an indirect immunofluorescence technique was used. Sections were incubated at room temperature with rabbit anti-mouse-immunoglobulin serum (RaM-Ig) (Nordic Laboratories, Tilburg, The Netherlands) for 20 min

as a first step, and washed three times with PBS (pH 7.8) for another 20 min. The RaM-Ig reacted with all classes of mouse immunoglobulins. Subsequently, the sections were incubated for 20 min with goat anti-rabbit-immunoglobulin serum conjugated to rhodamine (TRITC-GaR-Ig) (Nordic Laboraties, Tilburg, The Netherlands). Finally the sections were washed again with PBS for 20 min. All incubations were carried out in moisted chambers. The sections were then covered with buffered glycerol (9 parts glycerol and 1 part PBS, pH 7.8), a coverslip was applied and the edges were sealed with paraffin. The slides were examined with a Zeiss fluorescence microscope equipped with a vertical illuminator IV/F and an Osram HBO-50 mercury lamp as a light source. Black and white photographs were taken on Kodak Plus X Pan Films (22 DIN) with exposure times ranging from 30 to 60 seconds.

RESULTS

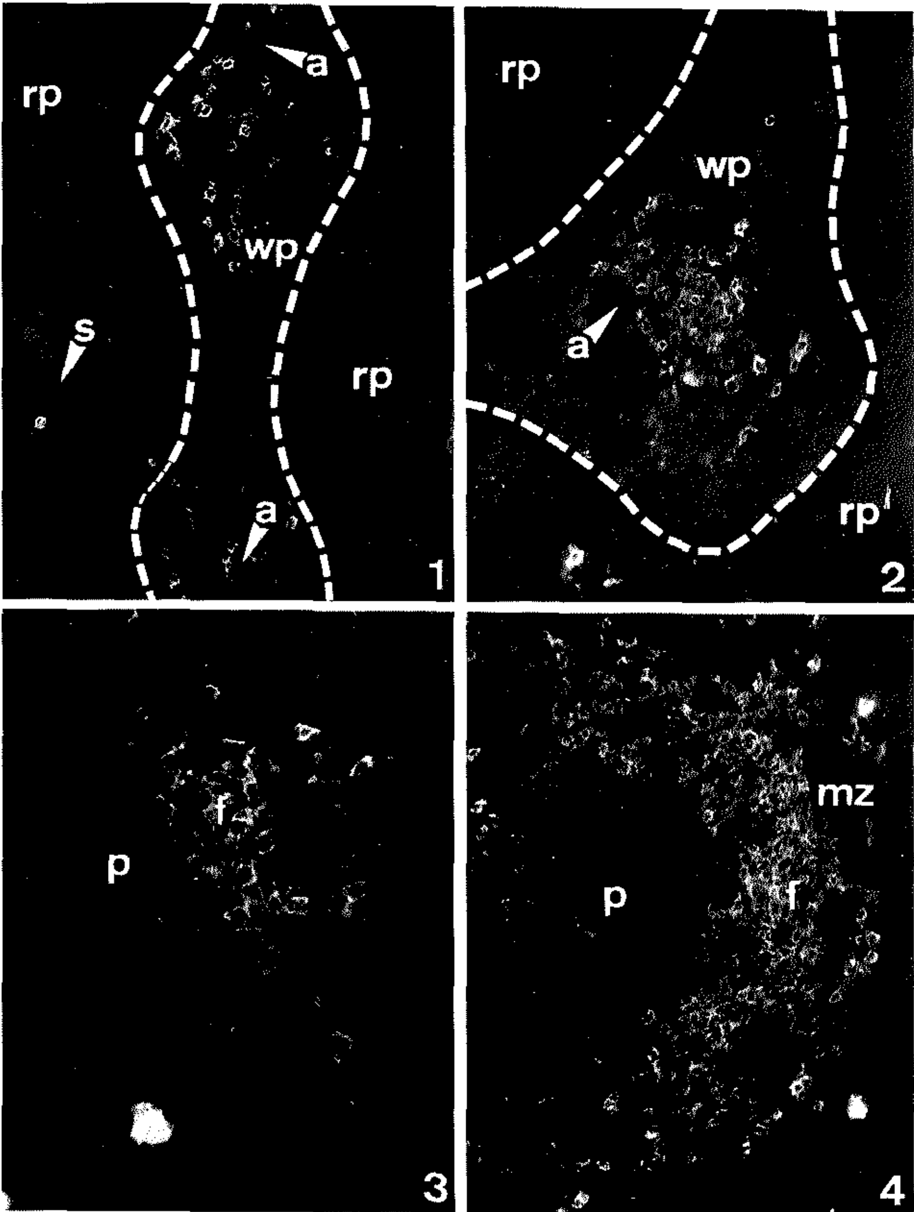
Recovery pattern in the spleen. The recovery of the B cell compartment was studied in the spleen, lymph nodes and Peyer's patches of mice at various times after irradiation and reconstitution with fetal liver cells. In the spleen of such mice only mature plasma cells, but no B cells, as identified by immunofluorescence using an anti-immunoglobulin serum as specific reagent, were found during the first week after irradiation and reconstitution. After that time, on day 8, the first cells with a typical B cell staining appeared. These B lymphocytes, recognizable by a thin, but very distinctly fluorescent margin, were found predominantly in the central part of the periarteriolar lymphatic sheath (PALS), located in a disperse way around the central arteriole (Fig. 1). Thereafter the number of B cells increased. Until day 11 the majority of these lymphocytes still occupied the central area of the PALS (Fig. 2). However, on day 11 in some sections the formation of small primary follicles in the peripheral part of the PALS could be observed (Fig. 3). From day 11 on the localization of B lymphocytes in the spleen was obviously in primary follicles in the peripheral PALS (Fig. 4). Nevertheless a distinct number of B cells remained

Fig. 1. Frozen section of the spleen of an irradiated and reconstituted mouse at day 8. B lymphocytes are located around the central arterioles (arrows) in the central part of the white pulp. a = central arteriole, s = sinus, wp = white pulp (inside the dotted line), rp = red pulp (x 200).

Fig. 2. Spleen of a mouse at 11 days after irradiation and reconstitution. The number of B lymphocytes increases, but these cells are still located in the central part of the white pulp around the central arteriole (arrow). a = central arteriole, wp = white pulp (inside the dotted line), rp = red pulp (x 220).

Fig. 3. White pulp of a mouse on day 11. B cells form small primary follicles at the periphery of the PALS. f = follicle, p = PALS (x 330).

Fig. 4. White pulp of a mouse at 13 days after irradiation and reconstitution. B lymphocytes occupy the peripheral part of the PALS and are located in distinct primary follicles. Hardly any fluorescent cells can be detected in the marginal zone. p = PALS, f = follicle, mz = marginal zone (x 200).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demonstrable in the central area of the PALS. At that moment, 13 days after irradiation and reconstitution, the marginal zone, surrounding the white pulp was still devoid of B lymphocytes (Fig. 4). At day 16, B lymphocytes dispersely appeared in the marginal zone (Fig. 5). These B lymphocytes showed the very bright fluorescence which is characteristic for B cells in that location (23). Furthermore, B lymphocytes were found in primary follicles, in the central part of the PALS and also very definitely in the red pulp of the spleen. From day 18 on the B cell compartment in the spleen of irradiated and reconstituted mice was qualitatively as in normal mice. B cells were located predominantly in large primary follicles in the peripheral part of the PALS (Fig. 6). These follicles were surrounded by a marginal zone, which consisted of B cells with a very bright fluorescence. In the central area of the PALS still B cells were found, but the number of these cells was decreased as compared with earlier stages of the recovery period. During the rest of the experimental period the number of B lymphocytes in the PALS remained at this level. On the other hand, the number of B lymphocytes in the red pulp of the spleen still increased during this period.

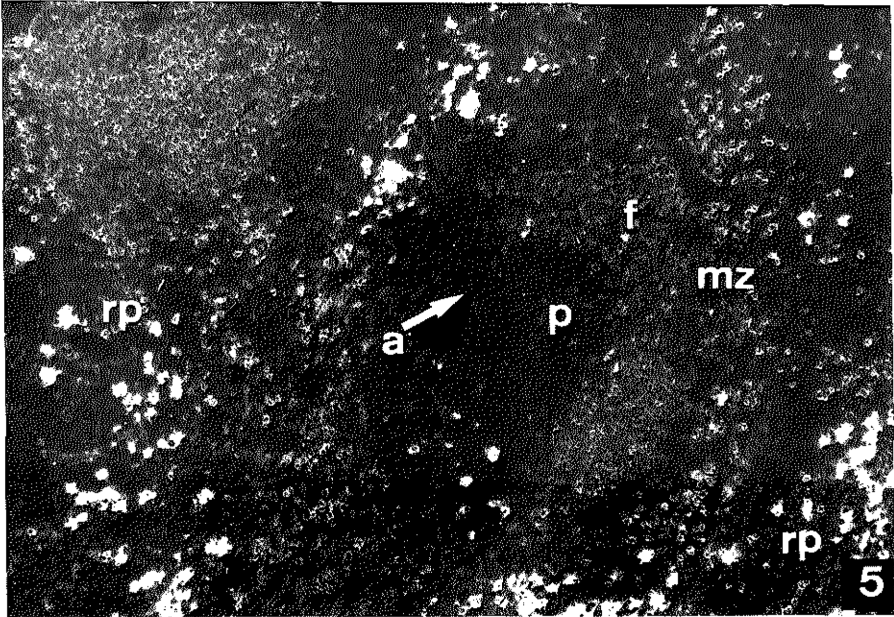
Although the B cell compartment in the spleen was qualitatively normal within 3 weeks, the quantitative recovery lasted longer. Normal spleen structures, comparable with the spleen of normal mice, were found around day 80 (Fig. 7). It also took some more time before the appearance of another typical B cell phenomenon, the germinal center reaction. Not before day 33 the first germinal center reaction could be observed in the spleen. Germinal centers were clearly recognizable as foci of non-fluorescent cells within positively stained follicles (Fig. 8). Furthermore, a cuff of strongly fluorescent dendritic cells was found at the peripheral site of the germinal center.

Recovery pattern in lymph nodes. B cell recovery in lymph nodes was studied in axillary, inguinal, brachial, and mesenteric lymph nodes. Since no differences were found between the results obtained from the various lymph nodes further data are combined and referred to as lymph nodes.

In lymph nodes the appearance of B cells took somewhat more time compared to the spleen. B lymphocytes in the lymhph nodes could be demonstrated for the first time 13 days after irradiation and reconstitution. They were located in very small numbers in the paracortical area. At the same time B cells occurred in small follicles in the outer cortex of the lymph nodes (Fig. 9). From day 13 till day 16 the

Fig. 5. White pulp of a mouse at 16 days. B lymphocytes are predominantly located in primary follicles at the periphery of the PALS. The first brightly fluorescent B cells are located in the marginal zone, which surrounds the white pulp. a = central arteriole, p = PALS, f = follicle, mz = marginal zone, rp = red pulp (x 120).

Fig. 6. White pulp of a mouse at 18 days after irradiation and reconstitution. B lymphocytes are located in large primary follicles at the periphery of the PALS. A large number of B lymphocytes showing a bright fluorescence is present in the marginal zone at this time. p = PALS, f = follicle, mz = marginal zone, rp = red pulp (x 120).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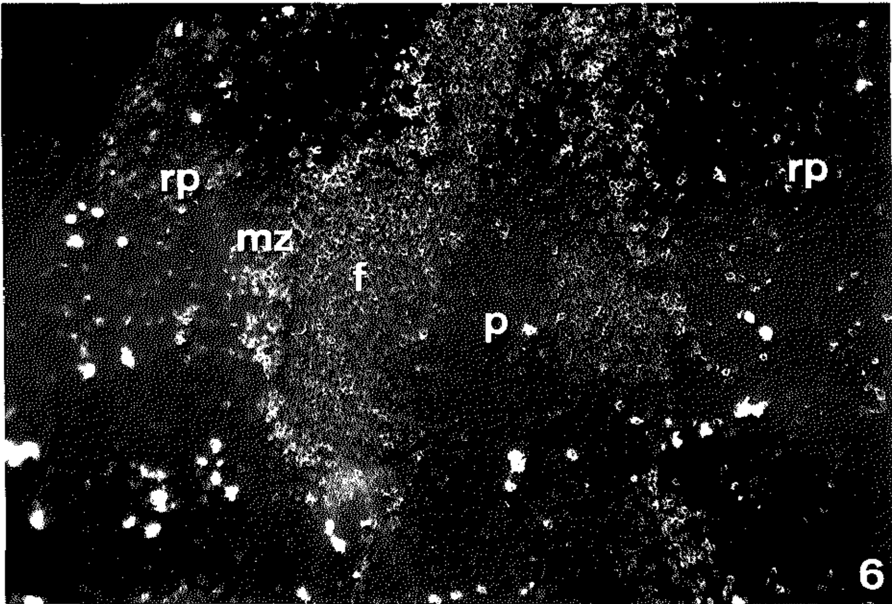
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number of B cells both in the follicles and in the paracortical area increased enormously. At day 16 already prominent follicles were present in the outer cortex (Fig. 10). These follicles were growing very fastly in size and around day 18 the complete outer cortex was occupied by a broad zone of follicles. At that time still a large number of B lymphocytes was scattered through the paracortical area.

Germinal centers in lymph nodes were not observed before day 25. From that time on the number of germinal centers increased fastly. Germinal center formation in lymph nodes seemed to preceed the comparable process in the spleen.

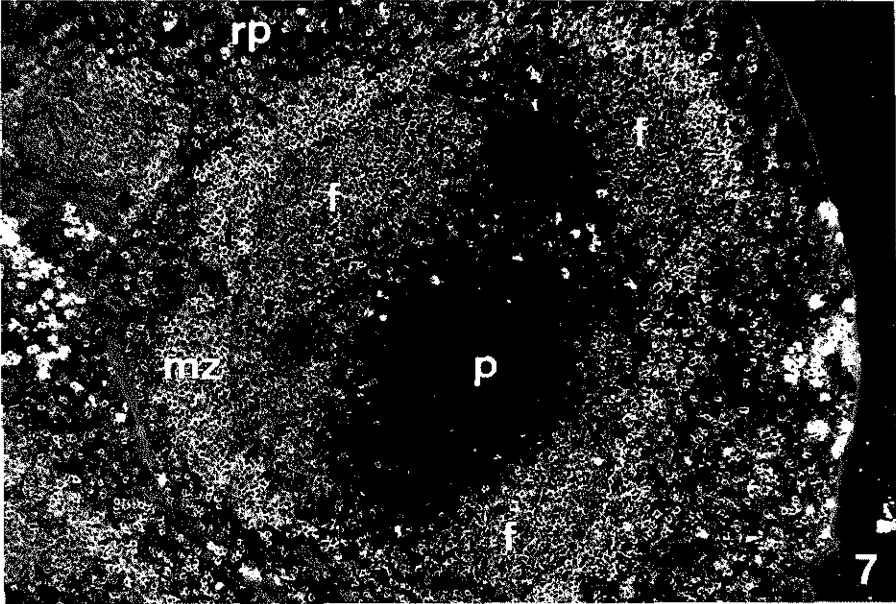
Recovery pattern in Peyer's patches. B cell recovery in the Peyer's patches after irradiation and reconstitution resembled very much the pattern as found in the lymph nodes. The first B lymphocytes in Peyer's patches were also found on day 13. Before that moment the size of the patches was severely reduced and only a few mature plasma cells remained. During the early recovery period after irradiation and reconstitution an enourmous diversity occurred in differentiation stage between the various patches on the intestine of a single mouse. The subsequently described recovery pattern for Peyer's patches concerns the patches, that recovered most rapidly.

B lymphocytes in large Peyer's patches were organized in small primary follicles and at first recognizable on day 13 (Fig. 11). The structure of these follicles, however, was very loose and a large number of B lymphocytes was scattered through the thymus-dependent area of the patches. After day 13 the number of B cells in the patches increased very fastly and on day 16 already large and full-grown follicles were found (Fig. 12). In these sections B lymphocytes were still dispersely located around the follicles. The organization of B cells in follicular systems continued and around day 22 distinct follicles could be recognized in all patches.

Germinal center formation in Peyer's patches developed in a similar way as in lymph nodes. Also at day 25 after irradiation and reconstitution the first germinal centers were found in the Peyer's patches.

Fig. 7. Spleen section of a mouse at 80 days after irradiation and reconstitution. Recovery of the B cell compartment is recovered completely at that time. p = PALS, f = follicle, mz = marginal zone, rp = red pulp (x 120).

Fig. 8. Germinal center reaction in the spleen of an irradiated and reconstituted mouse at 35 days. Note the "dendritic staining" of the peripheral part of the germinal center. The central part of the germinal center is Ig-negative. a = central arteriole, p = PALS, gc = germinal center, c = corona, mz = marginal zone, rp = red pulp. (x 200).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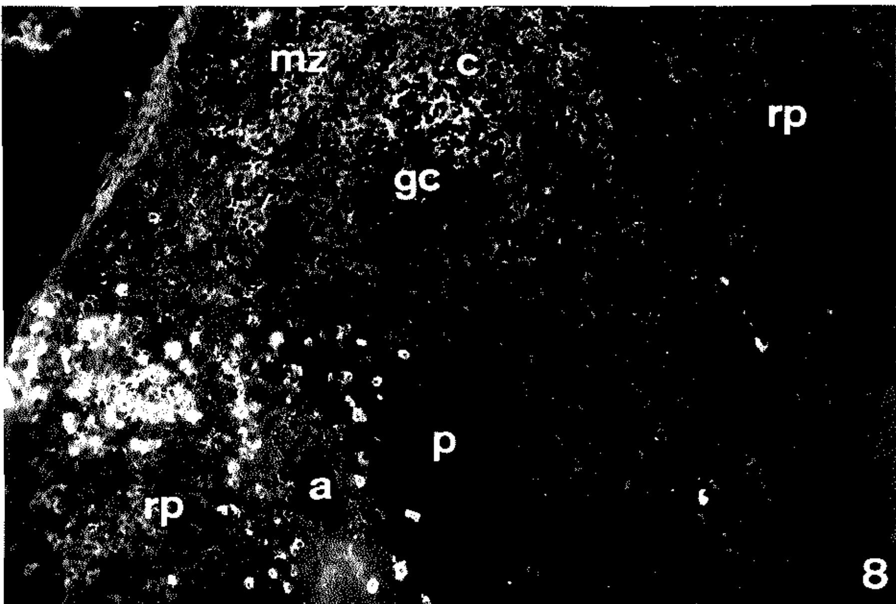
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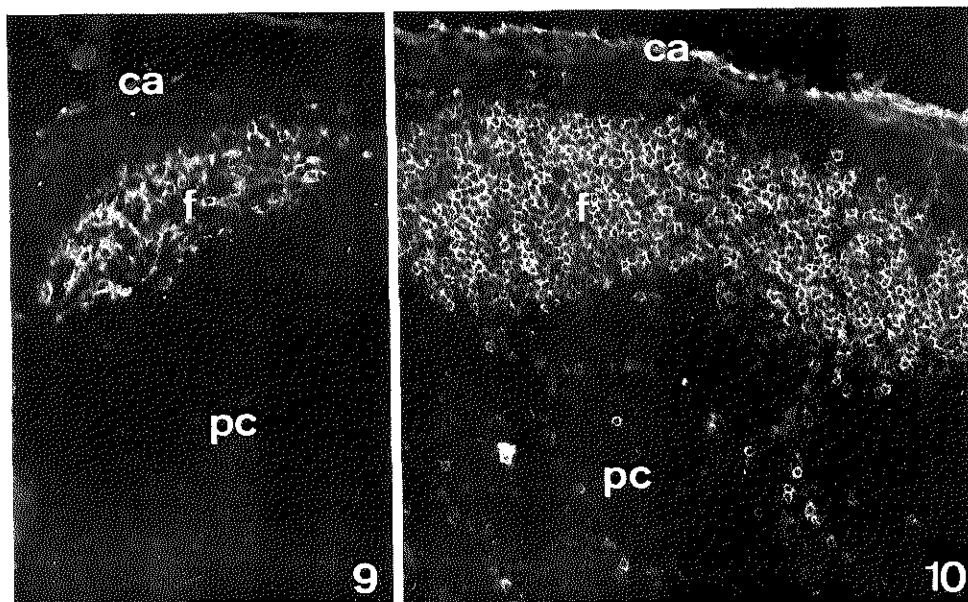

Fig. 9. Lymph node of a mouse at 13 days after irradiation and reconstitution. B lymphocytes are detectable in small primary follicles in the outer cortex of the lymph node. f = follicle, pc = paracortex, ca = capsule (x 200).

Fig. 10. Lymph node of an irradiated and reconstituted mouse at 16 days. B cells are located predominantly in large primary follicles in the outer cortex of the node. Scattered B cells are still present in rather large numbers in the paracortex. f = follicle, pc = paracortex, ca = capsule (x 200).

DISCUSSION

The present paper describes investigations on the recovery of the B cell system after irradiation and fetal liver reconstitution in mice. The first B lymphocytes, characterized by membrane fluorescence, appear in the spleen (24, 25). They are found in the central area of the PALS, which is a thymus-dependent compartment. This localization pattern resembles very much the localization of B lymphocytes in the spleen during ontogeny, as described by Friedberg and Weissman (26). These authors found, that B cells in the spleen of newborn mice are located in the immediate perivascular area up to 3 days after birth. At that stage B cells are separated from the blood vessels and accumulate in follicular systems at the periphery of the PALS. Friedberg and Weissman argue that this event of separation and accumulation is caused by the tremendous influx of thymus-dependent lymphocytes at that moment in the spleen of newborn mice, thereby simply pressing the already present

B lymphocytes to the periphery of the white pulp. However, in view of the results recently reported by Sprent (27), Nieuwenhuis and Ford (8), and Van Ewijk and Van der Kwast (28), functional properties of the B lymphocytes may underlay the localization pattern of B cells in the PALS which occurs both in young mice and in irradiated and reconstituted mice. These authors investigated the migration pathway of B lymphocytes from various sources and maturation stages through the spleen in animals with fully developed thymus-dependent areas. They found that B cells on their way to the follicles traversed the PALS. We suggest therefore, that the initial occurrence of B lymphocytes in the central area of the PALS after irradiation and fetal liver reconstitution is due to the migrational properties, which are already present in these, probably immature, B cells.

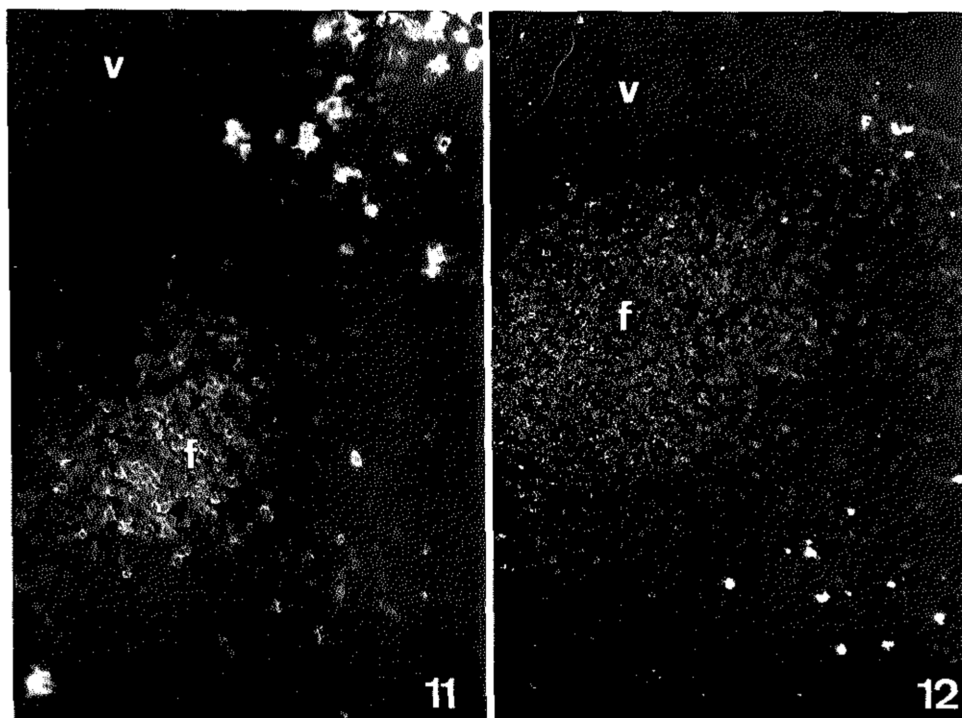

Fig. 11. Peyer's patch of a mouse at 13 days after irradiation and reconstitution. B lymphocytes are located in small disperse primary follicles, which are situated near the wall of the intestinal lumen. f = follicle, v = villi (x 200).

Fig. 12. Peyer's patch of a mouse at day 16. B cells are predominantly organized in large primary follicles, which are located near the wall of the intestinal lumen. f = follicle, v = villi (x 120).

It is remarkable, that at a stage, at which B cells are rather young and probably do not have the ability to start an immune response (25), they nevertheless follow the B lymphocyte migration pattern as found for a more mature (8, 28) and a recirculating (27) B cell population. Van Ewijk et al. (23) concluded from experiments, in which the cellular events during the primary immune response against sheep red blood cells (SRBC) were studied in the spleen of germ-free mice, that migration of B lymphocytes through thymus-dependent areas provides the opportunity for B-T cell cooperation during the humoral immune response. Obviously B cell migration is regulated by properties of B lymphocytes, which are already present in a very early differentiation stage and which are specific for B lymphocytes. On the other hand, reticulum cells in the PALS may also contribute to the recognition process of B cells in this area. Since *in vitro* destruction of membrane components on cells results in a changed homing pattern after transfusion of such treated cells into experimental animals (29), it is most likely, that also in the lymphoid system membrane determinants are involved in the migrational properties of B lymphocytes. Indeed Durkin et al. (30) found that pretreatment of B cells with an anti-immunoglobulin serum *in vitro* inhibited B cell traffic and subsequent homing in follicles after injection of these cells. They argue, however, that these results do not necessarily imply a direct role of surface Ig in this B cell traffic and suggest that C3 and Fc receptors are involved in the process of local B lymphocyte retention.

The scattered occurrence of B lymphocytes in the PALS and the disperse structure of the early follicles also suggests, that the appearance of B cells in these areas in the spleen is dependent on an influx of B lymphocytes from another organ, rather than on a clonal expansion *in situ*. The most likely place of origin of these immigrating B cells is the bone marrow, since quantitatively only this organ can be responsible for the number of B cells appearing in the spleen at that time after irradiation and reconstitution (25).

The first Ig-positive B lymphocytes are always found in the spleen of irradiated and reconstituted mice a few days before they can be demonstrated in the bone marrow (24, 25; also in these experiments, data not shown). Therefore, the appearance of recognizable amounts of surface Ig on these lymphocytes may occur somewhere on the way from production in the marrow until detection in the PALS in the spleen. This maturation of B cells probably occurs just before leaving the bone marrow, since at the time the first B cells can be demonstrated in the PALS also a few individual B lymphocytes are found in the red pulp of the spleen. This suggestion is in accordance with the results of Osmond and Nossal (31) and of Ryser and Vasalli (32), who showed that in the bone marrow of normal mice the amount of Ig-molecules on the surface of lymphocytes varied enormously between individual lymphocytes. They found that only 30 per cent of the marrow lymphocytes showed an Ig density comparable with Ig-bearing lymphocytes in spleen and lymph nodes. The remaining 70 per cent of the marrow small lymphocytes showed a lower density of surface-Ig varying between just demonstrable and not detectable. In another set of experiments these authors (32, 33) showed that during *in vitro* culture Ig-negative

lymphocytes in the marrow acquired increasing amounts of surface Ig during maturation. It may be concluded that the appearance of the first B lymphocytes in the spleen after irradiation and reconstitution in the central area of the PALS does not contradict the hypothesis, that the first part of the B cell differentiation in irradiated and reconstituted mice takes place in the bone marrow (15).

Already at about two weeks after irradiation and reconstitution the marginal zone which surrounds the lymphoid system in the spleen, becomes populated with B lymphocytes. These B cells show the same type of bright fluorescence as has been described in an earlier paper from our laboratory (22). Since there is both direct (31, 34) and indirect (35, 36) evidence that B memory cells have a higher density of Ig-receptors, we suggest that the more strongly fluorescent B cells in the marginal zone represent a distinct subpopulation of B lymphocytes, probably B memory cells. Although the exact mechanism of germinal center reactions is still unclear, data are reported in the literature (37, 38), which indicate strongly that this phenomenon is related to the formation of B memory cells. On the other hand, it is obvious from the data presented in this paper, that the appearance of the brightly fluorescent lymphocytes in the marginal zone distinctly preceeds germinal center reactions not only in the spleen, but also in lymph nodes and Peyer's patches. Consequently, if these strongly fluorescent lymphocytes in the marginal zone represent indeed B memory cells, the formation of B memory cells can occur without germinal center formation. In this respect some experiments performed in congenital athymic nude mice may be of importance. Intravenous injection of nude mice with a moderate dose of the thymus-independent antigen lipopolysaccharide (LPS) of *Escherichia coli* results in an excellent B memory response against this antigen (Benner and Van Oudenaren, to be published). Nevertheless it is almost impossible to demonstrate germinal center reactions in these mice after such a dose of LPS (Thorbecke, personal communication). These results suggest that B memory cells indeed can be generated without concomitant germinal center formation. The generation of B memory cells during the first phase of the recovery period probably took place in primary follicles. On the other hand, it cannot be overruled, that in a later stage of the recovery period as well as in normal mice, B memory cells are generated more effectively in germinal center reactions.

The recovery of the B cell compartment in the other tested lymphoid organs, lymph nodes and Peyer's patches, started later than in the spleen. A localization of the first B lymphocytes in Peyer's patches is difficult to establish. Usually B cells were found in small disperse primary follicles and scattered in the thymus dependent compartment at the same time. This is probably due to the fact that only large patches could be detected on the intestine and were used for subsequent tissue preparation in the first phase of the recovery period.

In the lymph nodes the first B lymphocytes are found in the paracortex. Similarily to the spleen B lymphocytes in the lymph nodes also follow the typical migration pathway through thymus-dependent areas (28). In contrast to the spleen the reconstitution of B cell compartments in lymph nodes and Peyer's patches into

large primary follicles proceeds very fastly. Within a few days after the appearance of the first small follicles full grown primary follicles could be demonstrated in all sections of these organs. This is probably an indication that B cell recovery in these organs is not dependent on an influx of similarily immature B cells as in the spleen. It is more likely that B lymphocytes invading lymph nodes and Peyer's patches around the third week after irradiation and reconstitution are part of a more mature cell population, possibly derived from the spleen. Accordingly, Röpke and Hougen (39) found, investigating the turnover and lifespan of small lymphocytes in nude mice, that B cells in lymph nodes are predominantly immigrated long-lived lymphocytes. Sprent (27) showed that after isogeneic transfer of radio-labeled B lymphocytes, obtained by thoracic duct drainage of nude mice the infused cells predominantly migrate towards the spleen. Subsequently a secondary migration from the spleen to lymph nodes occurred, which proved to be quantitatively important, although comparatively slow. Perhaps this slow tempo of redistribution is involved in the time difference between the start of the recovery of the B cell population in spleen and lymph nodes after irradiation and reconstitution. Another factor, that may be important in this view, might be the "saturation effect" as introduced by Bell and Shand (40). These authors found that saturation of irradiation-depleted lymphoid tissue with lymphocytes is an important factor regulating lymphocyte traffic.

The data presented in this paper agree with the hypothesis that the first part of the B cell differentiation in lethally irradiated and reconstituted mice occurs in the bone marrow. Thereafter, these rather immature B lymphocytes migrate probably towards the spleen for further maturation. Although these cells are still maturating, they follow the migrational pathway of mature B lymphocytes through thymus-dependent areas. From the time of appearance of germinal centers in the various organs we can conclude, that these structures are not essential for the early, antigen-independent recovery of the B cell population after irradiation and reconstitution. Furthermore, brightly fluorescent B lymphocytes, probably B memory cells, appear in irradiated and reconstituted mice in the marginal zone in the spleen after the formation of primary follicles, but before germinal center reactions could be observed. The sequence of these events suggests, that in the early stage of the recovery period germinal center formation is also not essential for the generation of the population of strongly fluorescent B lymphocytes in the marginal zone. In that stage this process probably occurs in primary follicles.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. Dr. O. Vos for his valuable criticism during the preparation of the manuscript. Mr. J. Fengler is acknowledged for his skilful photographic assistance and Miss C. Clerkx for typing the manuscript.

This investigation is part of a project program on the regulation of haemopoiesis, subsidized by the Netherlands Foundation for Medical Research (FUNGO). J. Rozing was supported by the Interuniversitary Institute for Radiation Pathology and Radiation Protection (Leiden, The Netherlands).

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