Identification and Kinetics of Two Recently Bone Marrow-Derived B Cell Populations in Peripheral Lymphoid Tissues

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Received September 23, 1994; accepted December 20, 1994

In rats, the glycoprotein Thy-1 is expressed on recently bone marrow (BM)-generated B cells but not on mature recirculating follicular (RF) B cells. Here we demonstrate that Thy-1* B cells consist of two phenotypically distinct, but developmentally related, populations: a population of newly formed (NF) B cells (IgM⁺⁺, IgD⁺⁺) that give rise to the second, less immature, Thy-1* population of so-called early recirculating follicular (ERF) B cells (Thy-1⁺ IgM⁺⁺ IgD⁺⁺) cells. These cells ultimately develop to RF-B cells (Thy-1⁺ IgM⁺⁺ IgD⁺⁺). Kinetic studies reveal that in absolute numbers per day most cells die at the transition of NF-B cells in the BM and those in the periphery: less cells die at later stages of B cell differentiation. Given the notion that this cell loss is not random, we speculate that NF-B cells and ERF-B cells may represent crucial steps during peripheral B cell development and their selection. Identification of their unique phenotype makes it possible to evaluate their roles in development of the antibody repertoire.

INTRODUCTION

Throughout the life of mammals, bone marrow (BM) is the primary site where huge numbers of B cells are generated each day from stem cells through a series of well-defined events that include rearrangement of immunoglobulin genes, proliferation, and differentiation. According to the current view of B cell development, pro-B cells (μ heavy chain negative) give rise to pre-B cells, (containing cytoplasmic μ chains), and finally to newly formed B cells (NF-B) expressing complete IgM molecules on their cell surface (for review see, e.g., Refs. 1, 2). NF-B cells differentiate to mature, long-lived recirculating B lymphocytes that express relative low levels of IgM and high levels of IgD (recirculating follicular, RF, B cells). The number of NF-B cells generated in the BM each day largely exceeds the number of cells that actually become incorporated into the peripheral B cell pool, and many of the newly generated cells will die (3–10). Recent evidence indicates that this cell loss is probably not random, but is the result of a ligand-driven selection process in which B cells are positively and/or negatively selected on the basis of the expression of their V region genes (11–19). Studies with Ig transgenic mice further suggest that this selection of B cells is not limited per se to BM, and that part of this process may occur in the periphery (17). Knowledge of the differentiation stages between NF-B cells in the periphery and mature follicular B cells is crucial to understanding selective events in vivo. NF-B cells appear to leave the BM since these cells have been found in blood and peripheral lymphoid tissues in rats and mice (20–24). In mice, NF-B cells express high levels of IgM (IgM⁺⁺) and very low levels of IgD (IgD⁻⁻) (24–27), in combination with high levels of heat-stable antigen (HSA) and low levels of B220 (CD45R) (24). However, this phenotype is also shared with other splenic B cells (24, 39). In rats, NF-B cells have a unique and distinct phenotype: in addition to their IgM⁺⁺IgD⁻⁻ phenotype, NF-B cells, but not MZ-B cells, express high levels of the determinant recognized by monoclonal antibody HIS24 (CD45R) (22, 23). Hunt et al. (28) and Crawford and Goldschneider (20) demonstrated more than a decade ago that “immature” B cells in rats express the Thy-1 antigen, while “mature” B cells are Thy-1-. In line with these observations we have shown recently that Thy-1⁺ B cells are rapidly replaced by dividing precursor cells, while Thy-1⁻ B cells are long-lived cells (9). Importantly, these studies also revealed that about 80% of the B cells die at the transition of Thy-1⁺ (immature) to the Thy-1⁻ (mature) B cell stage. This finding supports the notion that immature B cells are a crucial stage during 1990 Academic Press, Inc.
virgin (i.e., nonmemory) B cell development, during which the selective recruitment into the long-lived recirculating B cell pool takes place.

Although we showed that NF-B cells (defined as IgM⁺ IgD⁺ H24⁺ cells) are present in BM, blood, and spleen, but not in LN (22, 23), Thy-1⁺ B cells (immature B cells) were clearly found in all lymphoid tissues including LN (20, 28). These findings suggest that NF-B cells (IgM⁺ IgD⁺ H24⁺ cells) constitute only a fraction of the immature B cell pool. In the work described here we therefore define the phenotype of rat B cells in terms of IgM, IgD, H24, and Thy-1 expression using multicolor FCM analysis. In essence, we provide evidence for the existence of two developmentally related Thy-1⁺ B cell subpopulations in the periphery that are recent emigrants from the BM. In addition to NF-B cells, a previously unknown B cell subpopulation could be detected, which resembles mature, RF-B cells (IgM⁺ IgD⁺), but, in contrast, express high levels of Thy-1. These cells, present in all lymphoid tissues, are called early recirculating follicular (ERF) B cells. In combination with reconstitution and kinetic studies we propose a model of virgin B cell differentiation in rat in which NF-B cells are generated in the BM, migrate to blood and spleen, and give rise to ERF-B cells. We further suggest that these ERF-B cells subsequently differentiate to long-lived, mature RF-B cells.

MATERIALS AND METHODS

Animals

Lewis, AO, PVG, and BB rats were bred in the Department of Histology and Cell Biology or in the Central Animal Facility of the University of Groningen. Rats were housed under conventional conditions in the Department of Histology and Cell Biology. Rats were used at an age between 3 and 6 months. All rats were male except for AO rats used in transfer experiment using thoracic duct lymphocytes (TDL).

Antibodies

The following mouse anti-rat monoclonal antibodies (mAb) were used: HIS40 (anti-IgM) (22), HIS24 (directed to an epitope of CD45R) (29), HIS14 (directed to an epitope of CD45R) (29), MARD (anti-IgD, a kind gift from Dr. H. Bazin, University of Louvain, Brussels, Belgium), HIS51 (anti-Thy-1) (30), and OX19 (anti-CD5) (31). Mouse anti-BrdU mAb (IgG, isotype) was a generous gift from Dr. L. de Leij (University of Groningen, Groningen, The Netherlands). FCM analysis antibodies were conjugated with biotin, FITC, PE, or allophycocyanin (APC) as described (32). APC and PE were cross-linked with SMCC (succinimidyl 4-N-maleimidomethyl) cyclohexane carboxylic anhydride, Pierce, Rockford, IL) to the hinge region sulfhydryl of dithiothreitol-reduced antibodies (method modified from Ref. 33). Biotin-conjugated antibodies were revealed by streptavidin-allophycocyanin (Gibco/BRL, Gaithersburg, MD). Polyclonal goat anti-mouse IgG, conjugated to rhodamine (TRITC) was used to detect anti-BrdU on cytoplasm. All antibodies were appropriately diluted in phosphate-buffered saline (PBS) and centrifuged before use (10 min, 12000 × g) in a Biofuge A (Heraeus, Osterode, Germany) to remove aggregates.

Preparation of Cell Suspensions

Single-cell suspensions from spleen and cervical lymph nodes (CLN) were prepared by mincing tissue fragments between the frosted ends of microscope slides in Dulbecco’s A + B medium containing 5% newborn calf serum (NCS) (DAB/NCS) (Gibco Europe, Glasgow, Scotland). Large fragments were removed by passing the suspension through a nylon mesh. BM cells and peripheral blood cells were isolated as described previously (9). Erythrocytes in spleen cell suspensions and in the buffy coat were lysed using NH4Cl. Thoracic duct lymphocytes (TDL) were collected by cannulating the thoracic duct according to Ford and Hunt (34). The first overnight collection was taken and cells were washed twice before analysis or transfer.

BrdU Labeling

Pulse labeling. BrdU (Sigma, St. Louis, MO) was dissolved in 0.4% ammonia in distilled water to a concentration of 250 mg/ml, diluted with nine parts PBS (1×), and injected iv into rats (n = 3) at a dose of 50 mg/kg body wt (35). Rats were killed 60 min later.

Continuous labeling. Rats were given BrdU in their drinking water (1 mg/ml) for up to 5 days. To overcome the lag time in oral BrdU uptake, in addition, rats were injected ip six times with BrdU (50 mg/kg body wt) during the first 40 hr with an interval of 8 hr. BrdU-labeled cells are detectable in the BM less than 1 hr after the injection. Drinking water was protected from light during the experiment and was changed every 2 days.

Transfer Experiments

BM transplantation experiments were carried out using PVR rats. Rats were near- lethally irradiated with 8.2 Gy and reconstituted with syngeneic BM (1 × 10⁷ cells) 1 day later. Reconstituted rats were analyzed at time intervals varying between 1 day and 6 weeks after transfer. In a second set of experiments, 1 × 10⁷ TDL from AO rats were transferred 1 day after irradiation (8.2 Gy) into AO recipients. These animals were analyzed 1 week later.

Flow Cytometry Analysis and Cell Sorting

Multi-color FCM analyses were carried out as described previously (22). Briefly, 500,000 cells were
FIG. 1. FCM analysis of spleen and LN from a PVR rat after staining with a mAb directed to Thy-1 (HIS51) conjugated to PE, in combination with an anti-pan B cell mAb (HIS14) conjugated to APC. Data were taken from 20,000 cells and are presented as dot plots from cells with lymphoid forward and sideward scatter profiles.

stained in round-bottom microwell plates on ice with a combination of different mAbs conjugated to fluorescein, phycoerythrin, allophycocyanin, or biotin at predetermined optimal concentrations. After washing in sodium azide containing staining medium (DAB/NCSS), biotinylated antibodies were revealed by streptavidin-allophycocyanin as a second-step reagent. Finally, cells were washed, resuspended in 200 μl staining medium and analyzed or sorted on a dual laser Epics-Elite flowcytometer equipped with a gated amplifier ( Coulter Electronics, Hialeah, FL). During sorting, small-cell aggregates and doublets were disregarded by setting an additional sort gate on the FSC peak versus FSC integral ratio signal.

**BrdU Detection and Fluorescence Microscopy: Cell Counts**

After cell sorting, cells were cyt centrifuged and fixed in ice-cold ethanol-acetic acid (95:5 v/v) for 12 min. To detect BrdU incorporation, cells were subsequently dehydrated for 15 min in 2 M HCl at 37°C and incubated with anti-BrdU mAb followed by TRITC-conjugated goat anti-mouse IgG. Cytospin preparations were mounted in Citifluor AF1 (Citifluor, London, UK) to prevent fading of fluorescence. An epifluorescence microscope (Leitz, Wetzlar, Germany) equipped with an HBO 100-W mercury lamp and appropriate filter set (Ploemopak N) for excitation and emission of TRITC were used for analysis. Two-hundred fifty to 500 nucleated cells of each cell fraction were scored for the presence of nuclear BrdU incorporation (TRITC-positive cells).

**RESULTS**

**Two Distinct B Cell Subsets Expressing the Thy-1 Determinant**

FCM analysis of single-cell suspensions of rat spleen and LN with a pan B cell mAb (HIS14) in combination with a mAb to Thy-1 (HIS51) shows the expression of high levels of Thy-1 determinant on a subpopulation of B cells (Fig. 1). These Thy-1+ B cells account for approximately 20% of the B cells in spleen and 10% of the B cells in LN. Most B cells in these organs, however, do not (or at very low levels) express Thy-1.

Two-color FCM analysis of spleen cell suspensions for the expression of IgM and Thy-1 shows heterogeneity in levels of IgM, ranging from dull to bright, on Thy-1+ B cells (Fig. 2). Since B cells expressing low levels IgM (IgMlow) express high levels of IgD and vice versa (22, 23), B cell subpopulations are better resolved by simultaneous staining for IgM, IgD, and Thy-1. As we show in Fig. 3 by three-color FCM, Thy-1+ B cells are found among both the IgMhigh, IgDlow and IgMlow, IgDhigh splenic subpopulations. The relative frequencies of these two Thy-1+ B cells among these two subpopulations vary somewhat among rat strains (Table 1). In AO and PVR rats, about 30% of the IgMhighIgDlow cells and about 20% of the IgMlowIgDhigh cells in AO and PVR rats express Thy-1.

Previous studies demonstrated that IgMlow cells can also be subdivided on the basis of levels of expression of CD45R (B220), as recognized by mAb HIS24. Some of the IgMlow (and IgDhigh) cells express high levels of the HIS24 determinant, while the remainder expresses low levels. These cells represent GF-B cells and marginal zone B cells (MZ-B), respectively (22, 23). As we show in...
TABLE 1

Proportion of Thy-1 Positive and Thy-1 Negative B Cell Subpopulations in Lymphoid Tissues from Different Rat Strains

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Thy-1+</th>
<th>Thy-1-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(NF-B)</td>
<td>(MZ-B)</td>
</tr>
<tr>
<td>AO</td>
<td>3 (±1)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Blood</td>
<td>3 (±1)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Spleen</td>
<td>2 (±1)</td>
<td>4 (±1)</td>
</tr>
<tr>
<td>CLN</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>TDL</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>PVG</td>
<td>3 (±1)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Blood</td>
<td>3 (±1)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Spleen</td>
<td>3 (±1)</td>
<td>9 (±2)</td>
</tr>
<tr>
<td>Leu5</td>
<td>&lt;1</td>
<td>1 (±1)</td>
</tr>
<tr>
<td>Spleen</td>
<td>1 (±1)</td>
<td>13 (±2)</td>
</tr>
<tr>
<td>BB</td>
<td>4 (±1)</td>
<td>17 (±2)</td>
</tr>
</tbody>
</table>

*Numbers represent means (±SD) of the proportion of cells with lymphoid obuse and sideward scatter profiles. At least three animals were analyzed from each rat strain. Proportions of cells were determined by three-color FCM analysis of 20,000-30,000 cells after staining with anti-IgM conjugated to FITC, biotinylated anti-IgD and anti-Thy-1 coupled to PE. Biotin was revealed using streptavidin-APC as second-step reagent.

BM, blood, LN, and TDL (Fig. 2 and Tables 1 and 2). BM and blood contain both IgM+IgD+ and IgM+IgD- cells, whereas LN and TDL contain only IgM+IgD+ cells. In contrast with spleen, virtually all IgM+IgD+ cells in BM and blood express high levels Thy-1 (Table 1). These cells are also HIS24+ (data not shown) and therefore represent NF-B cells (22, 23). A minor fraction

TABLE 2

Phenotype and Anatomical Localization of B Cell Subpopulations in Rats

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>NF-B</th>
<th>ERF-B</th>
<th>RF-B</th>
<th>MZ-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IgD</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thy-1</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIS24</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Localization</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Blood</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Spleen</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>LN and TDL</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

*The levels of expression are based upon FCM analysis and are indicated as follows: ++, bright; +, dull; -, weak.

Fig. 3, FCM analysis for IgM, HIS24 (CD45R), and Thy-1 reveals that expression of Thy-1 on IgM cells coincides with levels of expression of HIS24 determinant: IgM"HIS24+ cells are all Thy-1+, while IgM"HIS24+ cells are all Thy-1-. Thus, based on the expression of IgM, IgD and Thy-1, and HIS24 we are able to distinguish four distinct B cell subpopulations in rat spleen (Table 2): (i) IgM"IgD"Thy-1+ cells, (ii) IgM"IgD"Thy-1- cells, (iii) IgM"IgD"Thy-1-HIS24+ cells, and (iv) IgM"IgD"Thy-1-HIS24- cells. These last two subpopulations represent the previously identified NF-B cells and MZ-B cells, respectively.

In addition to spleen, Thy-1+ B cells are also found in...
of IgM<sup>+</sup>IgD<sup>+</sup> cells in all organs studied, including LN and TDL (which lack IgM<sup>+</sup>IgD<sup>-</sup> cells) expresses Thy-1 (Table 1). IgM<sup>+</sup>IgD<sup>+</sup> B cells are recirculating cells which are located in situ in lymphoid follicles and were therefore previously called RF-B cells (36). Given the observation that IgM<sup>+</sup>IgD<sup>-</sup>Thy-1<sup>+</sup> B cells are present in TDL and probably represent a (developmentally) more immature population of cells (20, 28) (see below), we name this newly identified population of IgM<sup>+</sup>IgD<sup>-</sup> Thy-1<sup>+</sup> cells early recirculating follicular B cells, and limit the name RF-B cells to B cells with the IgM<sup>+</sup>IgD<sup>-</sup> phenotype.

**Thy-1-Positive B Cells Are the First to Return after Irradiation and BM Reconstitution**

To study a possible developmental relationship among the four B cell subpopulations (i.e., NF-B, ERF-B, RF-B, and MZ-B), we analyzed their reappearance following lethal irradiation and BM reconstitution. Three-color FCM analysis of reconstituted rats, 1 week after transfer, shows the presence of pro- and pre-B cells (i.e., sIgM<sup>+</sup>HIS24<sup>-</sup>Thy-1<sup>-</sup>) as well as pre-B cells expressing B cells in blood and spleen. Two weeks after transfer surface IgM expressing B cells can be detected. Virtually all of them have the phenotype of NF-B cells (IgM<sup>+</sup>IgD<sup>-</sup>Thy-1<sup>-</sup>). As we show in Fig. 4, ERF-B cells are clearly present in animals analyzed 1 week later (i.e., 3 weeks after BM reconstitution). In spleens from these reconstituted rats, more than 95% of the sIgM<sup>+</sup> B cells express Thy-1<sup>-</sup>, two-thirds of them representing NF-B cells and the remaining one-third representing ERF-B cells. Ultimately, Thy-1<sup>-</sup> B cells (i.e., RF-B cells and MZ-B cells) become manifest 4 weeks after BM transfer. The sequential reappearance of various B cell subsets suggests the following consecutive stages of B cell development: pro-/pre-B cells → NF-B cells (IgM<sup>+</sup>IgD<sup>-</sup>Thy-1<sup>-</sup>) → ERF-B cells (IgM<sup>+</sup>IgD<sup>-</sup>Thy-1<sup>-</sup>) → RF-B cells (IgM<sup>+</sup>IgD<sup>-</sup>Thy-1<sup>-</sup>) → MZ-B cells (IgM<sup>+</sup>IgD<sup>-</sup>Thy-1<sup>-</sup>) (see also Fig. 6).

**TDL Reconstitutes ERF-B Cells but Not NF-B Cells**

As reported in Table 1, more than 10% of the B cells in TDL are Thy-1<sup>-</sup>. All of these are ERF-B cells. One week after transfer of TDL into lethally irradiated rats, 6–10% of the cells with lymphoid scatter profile in spleen and LN are IgM<sup>+</sup>IgD<sup>-</sup> cells, while IgM<sup>+</sup>IgD<sup>-</sup> cells are absent (data not shown). Moreover, only rare Thy-1<sup>-</sup> B cells (<0.1%) of lymphoid cells) can be detected in spleen and LN of the repopulated rats. At most, these cells represent 2–4% of the IgM<sup>+</sup>IgD<sup>-</sup> cells in these organs. These findings are in accordance with a postulated developmental pathway, in which ERF-B cells differentiate to RF-B cells.

**Both Thy-1<sup>-</sup> B Cell Subpopulations Have a High Turnover Rate**

In a previous study we have shown that Thy-1<sup>-</sup> B cells in BM, blood, spleen, and LN are rapidly renewed while Thy-1<sup>-</sup> B cells are replaced at a much lower rate of approximately 1–2%/day (9). As shown above, Thy-1<sup>-</sup> B cells are subdivided into NF-B cells and ERF-B cells. To study more precisely the population dynamics of virgin B cell development, we established the kinetics of both Thy-1<sup>-</sup> B cell subpopulations.

The proliferative activity and renewal rate of each splenic Thy-1<sup>-</sup> B cell subpopulation was measured using BrdU incorporation. Splenic B cell subpopulations were isolated by three-color FCM cell sorting on the basis of their expression of IgM, IgD, and Thy-1 followed by...
FIG. 5. Disappearance of BrdU-negative Thy-1+ B cells in PIVG rat spleen during continuous administration of BrdU. Lines were obtained by linear regression of the values for the incidence of BrdU negative cells. Correlation coefficients (r) of the lines are 0.98 (NF-B cells) and 0.95 (ERF-B cells).

BrdU detection on cytopsin preparations. Pulse labeling studies show that 1 hr after a single iv injection of BrdU only rare (<0.2%) ERF-B and NF-B cells had incorporated BrdU. In contrast, 66% of the large pre-B cells in BM are labeled within this same time period (8). The very low S-phase indices for splenic ERF and NF-B cells indicate that both Thy-1+ B cell subpopulations are virtually devoid of cycling cells. Thus, we consider these stages of development as a simple transit compartment in which there is input, selection, and output, but no proliferation (38).

Continuous BrdU administration allows us to determine the renewal rates of splenic Thy-1+ B cell subpopulations. Rats were killed at several time points (3, 4 and 5 days) after the onset of the experiment. As we show in Fig. 5, approximately 90% of the FCM-sorted NF-B cells are labeled with BrdU within 5 days. The frequency of BrdU-containing NF-B cells follows an exponential relationship with time (38), resulting in a renewal rate of 72%/day (Table 3). ERF-B cells also become rapidly BrdU positive, albeit at a lower rate compared to NF-B cells. Five days after the start of the experiment about 70% of the ERF-B cells had incorporated BrdU. ERF-B cells also appear to be replaced in an exponential fashion with a renewal rate of 40%/day. Lag times, obtained by extrapolation of the linear regression lines (Fig. 5), are 49 hr for NF-B cells and 55 hr for ERF-B cells (Table 3). This difference is in accordance with our postulated model of rat B cell differentiation, in which a NF-B cells differentiate to ERF-B cells.

In marked contrast with these Thy-1+ B cell subpopulations, splenic B cells not expressing Thy-1 renew at a much lower rate: 5 days after the start of the experiment only 5% of the RF-B cells and 6% of the MZ-B cells are BrdU positive. Rat spleen harbors approximately 60 million Thy-1+ B cells (9) and as shown in Table 1, FCM analyses indicate that one-third of these cells are NF-B cells (i.e., 20 million cells); the remaining two-thirds are ERF-B cells. Based upon these absolute numbers, in combination with the respective renewal rates (72% and 40% per day), we estimate that 14 million splenic NF-B cells and 16 million splenic ERF-B cells are renewed daily (Table 3). The replacement in the entire splenic Thy-1+ B cell pool is thus 30 million cells per day, which is essentially the same as we estimated previously (27 million) (9).

DISCUSSION

The evidence presented here, together with previous data from this laboratory (8, 9, 22, 23, 29, 30, 37) and others (20, 28), leads to a coherent scheme of rat B cell generation and differentiation in the BM and periphery.

The model, which is shown in Fig. 6, provides for B cell genesis, differentiation, and vast expansion in the BM, followed by migration to the periphery where further differentiation to RF-B cells takes place without substantial expansion. It is only during subsequent humoral responses to antigen that further differentiation and expansion occurs in the periphery.

Hunt and Crawford previously demonstrated that Thy-1+ B cells in rats are recently BM-derived (or immature) B cells. Thy-1+ B cells dominate in BM and neonatal spleen and are the first B cells that reappear in regenerating peripheral lymphoid tissues following sublethal irradiation (20, 28). Recently, we provided additional evidence for this notion by studying the kinetics of Thy-1+ and Thy-1- B cells in lymphoid tissues using long-term BrdU administration (9). We showed that Thy-1- B cells are nondividing cells, but are all rapidly renewed, while Thy-1+ B cells are long-lived and are replaced only very slowly. In contrast to both of these populations, pro- and pre-B cells in the BM are actively dividing and rapidly renewed. Taken together, the data indicate that Thy-1+ B cells are recent descendants from proliferating B cell precursors located in the BM.

| TABLE 3
Renewal of Splenic Thy-1+ B Cell Subpopulations in the Rat |
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Cell compartment</td>
<td>Absolute numbers (10^6)</td>
<td>Renewal rate (%/day)</td>
<td>Renewal (10^6)</td>
</tr>
<tr>
<td>IgM+IgD-Thy-1+</td>
<td>(NF-B cells)</td>
<td>20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72</td>
</tr>
<tr>
<td>IgM+IgD-Thy-1-</td>
<td>(ERF-B cells)</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40</td>
</tr>
</tbody>
</table>

<sup>a</sup> PIVG/B male rats (13-15 weeks old) were continuously provided with BrdU.

<sup>b</sup> Renewal rates were obtained by linear regression of the values for BrdU incorporation (Fig. 5).

<sup>c</sup> Lag time, time between start of the experiment and appearance of the first BrdU cells, obtained by extrapolation of the linear regression lines (Fig. 5).

<sup>d</sup> Absolute numbers of Thy-1+ B cell subpopulations were calculated based upon their relative frequency in the spleen and the absolute numbers of Thy-1+ B cells in the spleen (Ref. 9).
In the present study we confirm and extend these observations and demonstrate that Thy-1 "B" cells consist of two phenotypically distinct subsets: NF and ERF-B cells (summarized in Table 2). NF-B cells express high levels of IgM and low levels of IgD, whereas ERF-B cells express low levels of IgM and high levels of IgD. We propose that these two Thy-1 "B" cell subpopulations represent developmentally related stages of virgin (nonmemory) B cell formation, in which NF-B cells, generated in BM, migrate to blood and spleen and differentiate to ERF-B cells. We base this scheme on several points. First, NF-B cells have a restricted distribution pattern: they are found in BM, blood, and spleen, but are absent from other lymphoid tissues. Apparently, these cells lack the essential homing receptors needed to adhere to the high endothelial venules in order to migrate into LN. ERF-B cells, in contrast, are widely distributed among all lymphoid tissues examined. The presence of ERF-B cells among TDL demonstrates that these cells have acquired the capacity to enter LN allowing them to recirculate. Second, reconstitution of lethally irradiated rats with BM shows that NF-B cells reappear first in regenerating spleen, shortly thereafter followed by ERF-B cells. Third, the first Thy-1 "B" cells identified in neonatal spleen are NF-B cells (F. G. M. Kroese et al., manuscript in preparation). Although both NF-B cells and ERF-B cells are present in spleen, this organ is probably not an obligatory site for this differentiation step. Frequencies of B cell subpopulations in blood and LN from neonatally splenectomized rats do not differ significantly from that of control rats when tested more than 3 months after surgery (N. K. de Boer, unpublished observations). It is thus possible that also in normal animals part of the differentiation from NF-B cell to ERF-B cells takes place in BM or in blood.

There are indications that ERF-B cells mature (directly) to RF-B cells. In BM reconstitution experiments, Thy-1 "B" cells reappear after ERF-B cells. A similar developmental pattern is observed during the ontogeny of the spleen (F. G. M. Kroese et al., manuscript in preparation). Furthermore, in lymphoid tissues taken from irradiated rats, repopulated with TDL (containing 5% ERF-B cells), ERF-B cells are virtually absent 1 week after transfer. This drop in frequency is in accordance with the renewal rate of ERF-B cells in normal animals (40%/day) and is expected if ERF-B cells differentiate to RF-B cells. Although, direct evidence is lacking, based upon the aforementioned findings we suggest the following virgin B cell differentiation pathway in rats: NF-B cells → ERF-B cells → RF-B cells.

Huge numbers of NF-B cells are produced in the BM each day (1, 2). This production significantly exceeds the renewal of the mature, peripheral B cell compartment. For example, in rats, 57 million NF-B cells are generated per day, while only 10 million are recruited into the mature B cells pool (9). Thus, the majority of NF-B cells (>80%) are eliminated during subsequent differentiation stages in the BM or periphery. The finding that Thy-1 "B" cells can be divided into two developmentally related immature B cell subsets allows us to further dissect the peripheral differentiation pathway in terms of their dynamics. In Fig. 6 we show a comprehensive view of the replacement of cells in each of the virgin B cells compartments that can be distinguished. We demonstrate that 14 million NF-B cells are replaced daily in the spleen (Table 3). Combining frequencies of NF-B cells with absolute numbers of nucleated cells (9), we conclude that >90% of peripheral NF-B cells are located in the spleen. Assuming that the renewal rate of NF-B cells in blood is similar to that in the spleen, the total renewal in the peripheral NF-B cell compartment is estimated to be 16 million cells per day. Given the renewal of 57 million NF-B cells in the BM per day (9), more than 40 million B cells (70%) die at the transition of the compartment of NF-B cells in the BM and that in the periphery.

ERF-B cells in spleen are renewed at a rate of approximately 16 million cells per day (Table 3). In peripheral tissues apart from spleen and blood Thy-1 "B" cells represent exclusively ERF-B. The turnover of these cells was estimated to be 6 million cells per day (9). Together with a small renewal in the blood this results in a total replacement in the extrasplicic peripheral ERF-B cell pool of 8 million cells. Considering all ERF-B cells as members of a single compartment of cells, the total renewal in this compartment would be 24 million (i.e., 16 + 8 million) cells daily (Fig. 6). However, since Thy-1 "B" cells are noncycling, the number of ERF-B cells that are replaced within this compartment should not exceed the number of cells that leave the peripheral NF-B cell compartment (16 million cells). This discrepancy may merely reflect inaccuracies in estimates of numbers of cells replaced in the NF-B cell and the ERF-B cell compartments. Alternatively, one can speculate that the
ERF-B cell compartment in the rat consists of two distinct compartments: a transient splenic ERF-B cell compartment that feeds the remaining extrasplicenic peripheral ERF-B cell compartment (Fig. 6). ERF-B cells eventually develop to the stage of mature virgin (Thy-1+) B cells, including both RF-B cells and MZ-B cells. Also during this differentiation step cell loss may be present. As shown previously, Thy-1+ B cells have a long life span and renew at a rate of 1-2% per day, implying a replacement of about 10 million cells (9), which is less than the number of cells replaced in the (total) ERF-B cell compartment.

In conclusion, in addition to the cell death observed during the generation of NF-B cells in the BM which may be associated to a large extent with the production of functional Ig molecules (1, 2, 4, 6, 16), many cells die upon subsequent virgin B cell development in rats, more than 80% of the NF-B cells die before these cells become mature, RF-B cells. As we have shown here (Fig. 6), most cells are lost between the stages of NF-B cells in BM and those in the periphery (57 million → 16 million). In terms of cell number, fewer cells die during subsequent differentiation stages leading to RF-B cells (16-24 million → 10 million). This cell death occurs most probably by apoptosis (18) and is associated with the selection process that operates to recruit cells into the mature, recirculating B cell pool (10-18). Both positive and negative selection mechanisms are thought to be involved in the establishment of the available B cell repertoire. Experiments by Gu et al. analyzing the usage of individual members of the J558 Vh gene family in (pre-) B cell subpopulations in mice, show that RF-B cells are (positively) selected cells (11). Transgenic mouse experiments by Nemazee and Burki (15) demonstrate that deletion (negative selection) of auto-reactive B cells, directed to a cell membrane-anchored autoantigen, occurs at the level of immature B cells. Also, in the anti-HEL/membrane-bound HEL double transgenic mouse system employed by Hartley and colleagues, antigen-reactive cells are deleted at the immature B cell stage (14, 18). Deletion of (auto-) antigen-reactive B cells may occur in the BM or in the periphery, depending upon the site where the autoantigen is expressed (15, 17). Together, these data indicate that immature B cells are subjected to selection and thus constitute a crucial phase in B cell differentiation. Our observation that many cells are lost between the NF-B cell compartment in BM and the RF-B cell compartment in the periphery is consistent with this view. The phenotypical identification of two subpopulations of B cell populations recently generated in the BM, i.e., NF-B cells and ERF-B cells, in rats allow for further dissection of the selective events which shape the peripheral B cell repertoire in normal animals and predicts candidate stages of the positive and/or negative selection process.

ACKNOWLEDGMENTS

The authors thank Astrid Wubben, Boels Meedendorp, and Susanne Stelling for technical assistance and Geert Mesander for help with FCM analysis. This work is supported by NATO collaborative Grant CRG 910185. A.B.K. acknowledges support from NIH Grant HD 01287 to L.A. Herzenberg.

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