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The rat B cell system: the anatomical localization of flow cytometry-defined B cell subpopulations*

Two-color flow cytometrical (FCM) analysis of rat peripheral lymphoid organs shows two distinct IgM/IgD-defined B cell subpopulations, similar to those of the mouse: a major population of cells expressing little IgM and high levels of IgD (population I) and a minor population of cells expressing high levels of IgM but little IgD (population III). In peripheral lymphoid organs population III cells are mainly found in spleen where they represent about 25% of the B cells; population III cells are almost absent from lymph nodes and Peyer's patches. In adult bone marrow and in neonatal spleen the majority of IgM/IgD-defined B cells (> 70%) are population III cells, similar to what is observed in the mouse. In contrast with mice, only a low proportion of the cells (1%) recovered from the peritoneal cavity are B cells, and most of them belong to population I.

Previously defined monoclonal antibodies (HIS22 and HIS24) to B cell forms of the leukocyte common antigen (CD45R) in combination with staining for surface IgM and surface IgD demonstrates a further heterogeneity of rat B cells by three-color FCM analyses. HIS22 labels most population I cells; population III cells and a small subset (about one third) of population I express only very low levels of the HIS22 determinant. HIS24 reacts with population I cells and subdivides population III into two subsets: about one third of splenic population III cells are brightly stained with this antibody whereas fluorescence of the remaining two-thirds is lower. The HIS24^{bright} population III cells likely are newly formed B cells since cells with this phenotype are the predominant surface Ig population found in adult bone marrow and neonatal spleen.

In tissue sections of lymphoid organs, HIS22- and HIS24-positive cells are mainly found in lymphoid follicles; splenic marginal zones are almost unstained. Combining immunohistological analysis with the FCM data, we therefore conclude that the small follicular B cells are in population I and marginal zone B cells are found in the HIS24^{dull} population III. The *in situ* localization of HIS24^{bright} population III cells and the HIS22^{dull} population I cells is not clear.

Based upon the relative expression of surface markers on B cells we propose a model of B cell differentiation in the rat in which a common precursor cell (the HIS24^{bright} population III cell), produced in the bone marrow, enters the spleen and leads to two distinct B cell differentiation pathways: one leading to marginal zone B cells and the other giving rise (through an intermediate cell type) to the majority of B cells, the small resting follicular B cells.

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Abbreviations: **MZ:** Marginal zone **GC:** Germinal center

1 Introduction

Immunohistological staining of tissue sections and multiparameter FCM analysis of single-cell suspensions have demonstrated heterogeneity in the phenotype of B cells. For example, in the adult murine spleen at least two subpopulations of B cells have been found by FCM: a relatively large population consisting of of IgM^{dull}IgD^{bright} cells which is called population I, and a much smaller population of IgM^{bright}IgD^{dull} cells which is called population III [1–3]. This last population also comprises the vast majority of B cells that belong to the Ly-1 B lineage, a separate lineage of B cells with distinct phenotypical, functional and anatomical properties (reviewed in [4]).

A similar heterogeneity is also seen in immunohistologic studies of tissue sections of spleen. In spleen, B cells are predominantly found in the white pulp, where they are localized in two distinct compartments, the lymphocyte follicle and the marginal zone (MZ). Especially in rats, MZ are easily identifiable and are supposed to be the largest

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splenic B cell compartment [5]. Immunohistological staining with anti-IgM and anti-IgD antibodies demonstrated that MZ cells (in rats and mice) are predominantly IgM^+IgD^- cells, in contrast to the follicular B cells which are IgM^+IgD^+ cells [6–8]. Functionally, MZ B cells may be involved in the immune response to T cell-independent antigens [5, 9]. It has been speculated that they may be related to murine Ly-1 B cells [4]. Despite the apparent similarities in the presence of two distinct IgM/IgD-defined subpopulations using FCM or immunohistology, the relationship between the anatomically defined and the FCM defined populations is not fully established.

We have recently developed a panel of mAb (HIS14, HIS22 and HIS24) to B cell-associated determinants in the rat [10]. Most likely all three antibodies are directed against epitopes of the B cell form(s) of the rat leukocyte common antigen (CD45R), similar to the B220 antigen in the mouse. Two of these mAb, HIS22 and HIS24, differentially stained the different splenic B cell compartments in immunohistologic studies; HIS22 stains the lymphocyte corona, but not (or weakly) germinal centers (GC) and HIS24 stains both the lymphocyte corona and the GC. Both antibodies stain only (very) weakly the splenic MZ. Here we use these antibodies together with anti-IgM and anti-IgD antibodies to explore further the rat B cell system by three-color FCM and to correlate FCM defined and anatomically defined B cell subpopulations.

2 Materials and methods

2.1 Animals

Male Fisher 344 rats were obtained from Simonsen (Gilroy, CA) and were kept under conventional conditions in the animal facility of the Veterans Administration Hospital, Palo Alto, CA, until use at the age of 3–4 months. Neonatal rats of either sex were used at 2 days, 10 days or 3 weeks of age.

2.2 Antibodies

Mouse anti-rat IgM mAb (HIS40; IgG_1) was generated in the Department of Histology and Cell Biology (University of Groningen, Groningen, The Netherlands) by fusing splenocytes from BALB/c mice immunized with rat B lymphoblasts with the murine myeloma cell-line X63Ag8 (F. G. M. Kroese, A. S. Wubbena and D. Opstelten, unpublished). Mouse anti-rat IgD mAb (MARD 3) was a generous gift from Dr. H. Bazin, Univerity of Louvain, Brussels, Belgium. The mAb HIS14, HIS22 and HIS24 to B cell-associated determinants of the rat were developed and characterized as previously described [10]. MRC OX19 a mAb against a 69-kDa rat Tcell antigen was kindly provided by Dr. A. F. Williams (Sir William Dunn School of Pathology, Oxford, GB; [11]). All antibodies were purified from ascites fluid using DEAE-Sephacel (Pharmacia, Uppsala, Sweden) ion-exchange chromatography [12]. Purified mAb were conjugated to FITC (Molecular Probes, Inc., Junction City, OR), biotin (Biosearch Research Chemicals, San Rafael, CA) or allophycocyanin (APhC) as described in detail [3, 12]. Texas red (Molecular Probes, Inc.) was conjugated to avidin D (Vector Laboratories, Inc., Burlingame, CA) as described [3].

2.3 Cell suspensions

Single-cell suspensions from cervical LN and spleen were prepared in cold RPMI staining medium (RPMI 1640, 4% newborn calf serum, 10 mM Hepes, 0.1% NaN_3) by mincing tissue fragments between the frosted ends of microscope slides and filtering through a nylon mesh. Peritoneal cells (PC) were collected after injection of ca. 15 ml cold RPMI medium into the peritoneal cavity of adult rats.

2.4 Immunofluorescence staining and FCM analysis

Three-color immunofluorescence staining of cells using FITC-, biotin- and APhC-conjugated mAb was carried out in RPMI medium as described in detail elsewhere [3]. Biotinylated antibodies were revealed by TR-avidin as second-step reagent. Dead cells were stained with propidium iodide (1 μg/ml) and this staining was added as a fourth color in our analysis. Cells were analyzed on an extensively modified dual laser fluorescence-activated cell sorter (FACS II: Becton Dickinson, Mountain View, CA) interfaced with a VAX 11/780 computer (Digital Equipment, Maryland, MA) as described [3]. For spleen cell suspensions, erythrocytes were gated out on the basis of their characteristic forward scatter. For each analysis data from 20000–30000 viable (*i.e.* propidium iodide-negative) cells were collected. Data are presented as 5% probability contour maps after gating out cells with high forward and obtuse scatter.

2.5 Immunoperoxidase staining

Cryostat sections of rat spleen were stained with mAb using an indirect immunoperoxidase technique as has been described elsewhere [10]. As second-stage antibody, peroxidase-conjugated polyclonal rabbit anti-mouse Ig (Dakopatts, Copenhagen, Denmark) was used.

3 Results

3.1 IgM and IgD define subpopulations of rat B cells similar to those in the mouse

Two-color staining of cell suspensions from rat peripheral lymphoid organs (spleen, LN and Peyer's patches) with anti-IgM and anti-IgD mAb shows that the predominant B cell population in rats is bright for IgD and dull for IgM (Fig. 1), similar to the predominant B cell subset (population I) in the mouse [1–3]. A second, smaller, subpopulation of B cells consisting of cells that express relatively little IgD but high levels of IgM, similar to population III defined in the mouse, is also present in rats.

Fig. 1 shows that, as in the mouse, population III cells are mainly found in spleen and are almost absent from LN and Peyer's patches. As shown in Table 1 population III cells account for $< 5\%$ of the IgM/IgD-defined B cells in LN and Peyer's patches. In adult rat spleen, population III cells repesents at most about 25% of the IgM/IgD-defined B cells. However, in neonatal rat spleen population III cells ($IgM^{bright}IgD^{dull}$) predominate: $> 70\%$ of all IgM- and/or IgD-positive cells from 2-day-old spleens and up to about 50% of the B cells from 3-week-old spleens are bright for

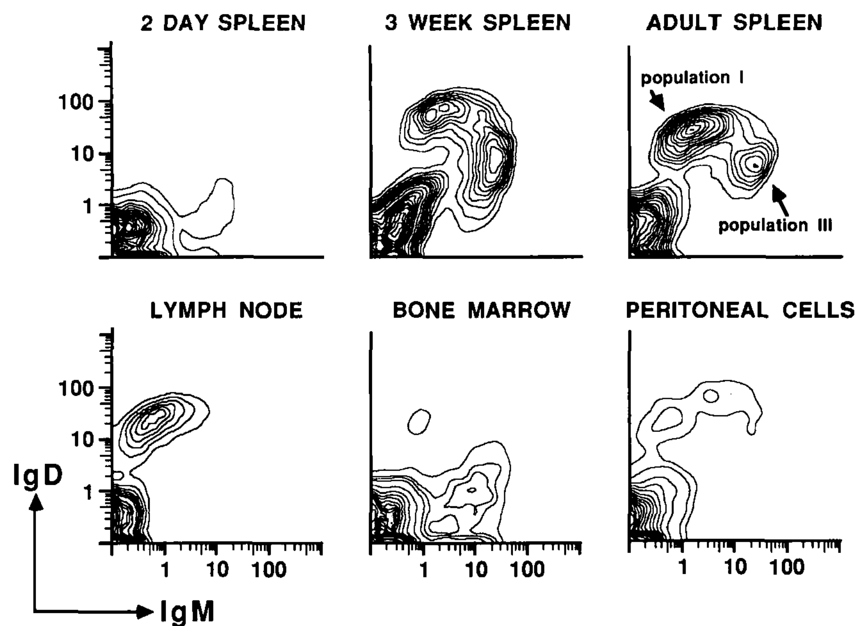


Figure 1. B cell subpopulations in rat tissues as revealed by two-color FCM analysis after staining with fluoresceinated anti-rat IgM (HIS40) and biotinylated anti-rat IgD (MARD 3), followed by avidin-conjugated to Texas red.

IgM and dull for IgD (Fig. 1). The modal expression of IgD on 2-day-old splenocytes is even lower than that on IgM^{bright} cells in adult spleen. Also in adult BM, many population III cells are present: about 75% of the sIgM/sIgD-defined B cells belong to population III.

In the mouse high numbers of B cells are found in the peritoneal cavity of normal adult mice. Most of the peritoneal B cells belong to a distinct lineage of B cells (termed the Ly-1 B lineage; [4]), most of which express low amounts CD5 (Ly-1) on their cell membrane. By contrast, in rats only about 10% of the cells recovered from the peritoneal cavity showed the typical forward and obtuse scatter of lymphoid cells, of these cells < 10% (*i.e.* 1% of total PC) expressed IgM and/or IgD (Fig. 1, Table 1). This low proportion of B cells in rat peritoneal cavity makes further FCM analysis of these peritoneal B cells difficult.

In the mouse, Ly-1 B lineage cells are also found in relatively high numbers in neonatal spleen and to a lesser extent also in adult spleen [4, 13]. In the rat the homologue for the murine Ly-1 (CD5) determinant appears to be recognized by mAb MRC OX19 [11]. However, two-color FCM analysis of rat adult lymphoid organs and neonatal spleen with anti-IgM and MRC OX19 could not reveal a distinct population of B cells expressing higher levels of the determinant recognized by MRC OX19 than the predominant B cell population. In fact it seems that in the rat all neonatal B cells express low amounts of the determinant recognized by MRC OX19 (data not shown). Thus, we could not yet establish unequivocally the presence of CD5 (Ly-1) B lineage cells in these rats.

3.2 HIS22 and HIS24 further subdivide rat B cell subpopulations

3.2.1 General remarks

Recently developed mAb (HIS14, HIS22 and HIS24) have been shown to recognize B cell-associated determinants (CD45R; [10]). By two-color FCM we confirmed here that the determinants recognized by HIS14 and HIS24 are also expressed, predominantly in low amounts, on a minor proportion (15%) of the peripheral T cells (defined as MRC $OX19^{bright}$ cells) whereas < 1% of the T cells are stained with HIS22 (data not shown).

APhC-conjugated HIS mAb were used in three-color analysis in conjunction with the anti-IgM and anti-IgD antibodies to further define rat B cells. HIS14 did not further subdivide population I and/or population III: virtually all B cells in the rat are bright for HIS14 and only 1%–2% of both population I and population III cells in peripheral lymphoid organs were HIS14 negative (or dull; Table 2). By contrast, using HIS22 and HIS24, marked differences in reactivity were seen with B cells from either of the two IgM/IgD-defined B cell subpopulations. In the mouse such a subdivision has not yet been identified with anti-CD45R (B220) antibodies.

3.2.2 HIS22 mainly reacts with the population I cells

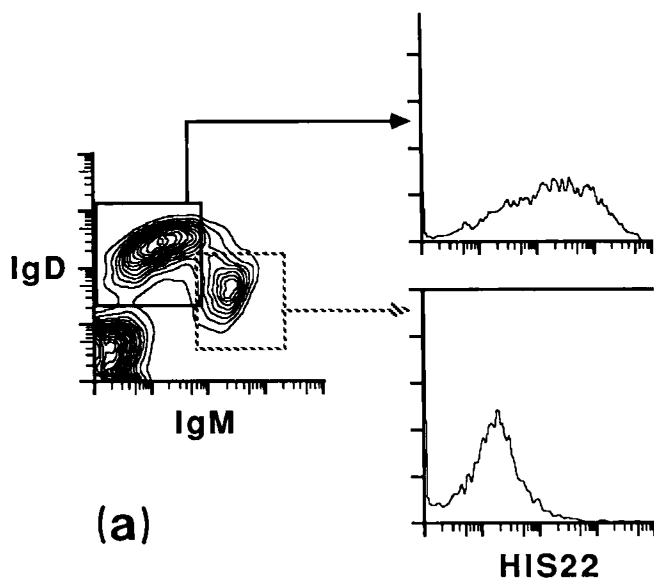
Fig. 2 shows the reactivity of HIS22 with the two IgM/IgD-defined B cell subpopulations (*i.e.* population I and III) in the spleen. The HIS22 staining pattern of population I or III cells is quite broad, ranging from negative to very bright, but population I is clearly enriched in $HIS22^{bright}$ cells and population III in $HIS22^{dull}$ cells. As is shown in Table 2, roughly two-thirds of the population I ($IgM^{dull}IgD^{bright}$) cells in the spleen are bright for HIS22. The vast majority (approximately 90%) of the population III ($IgM^{bright}IgD^{dull}$) cells, however, are dull for HIS22 (on average, about 20–25 times duller than the $HIS22^{bright}$ cells).

Also in the BM, where relative high numbers of population III cells are found, the cells that belong to this subpopulation are also $HIS22^{dull}$ (Fig. 3). Since these cells represent the majority of B cells in the BM, these ($HIS22^{dull}$) population III cells may represent newly formed B cells. Likewise, B cells from neonatal mice, which predominantly belong to population III, are mainly $HIS22^{dull}$ (Fig. 3).

Table 1. Proportion of IgM/IgD-defined B cell subpopulations in adult rat tissues[a)]

	Spleen	LN	Peyer's patches	Peritoneal Cavity
	$n = 7$	$n = 4$	$n = 4$	$n = 3$
Cells with lymphoid scatter profile	93 ± 3[b)]	97 ± 1	93 ± 1	10 ± 1
Lymphocyte subpopulations				
$IgM^{dull}IgD^{bright}$(population I)	31 ± 4[c)]	25 ± 6	41 ± 7	6 ± 1
$IgM^{bright}IgD^{dull}$(population III)	12 ± 3	1 ± 1	1 ± 1	2 ± 1
IgM^-OX19^+	40 ± 6	70 ± 8	37 ± 9	28 ± 2
IgM^+OX19^+	<1	<1	<1	<1

a) Two-color FCM analysis of cell suspensions stained with FITC-anti-IgM and with biotin-anti-IgD, biotin-MRC OX19 or APhC MRC OX-19. Biotinylated antibodies were revealed by Texas red-avidin.
b) Proportion of total viable (*i.e.* propidium-negative) cells.
c) Proportion of cells with lymphoid scatter profile.



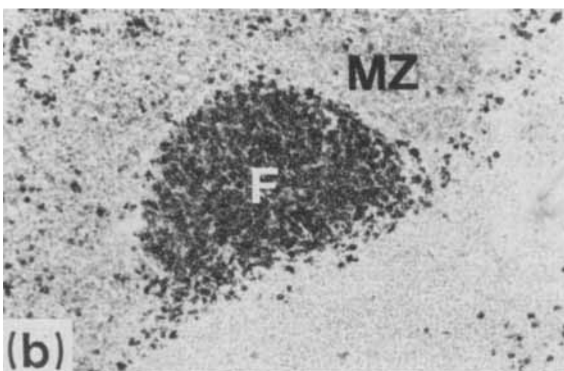


Figure 2. (a) Three-color immunofluorescence demonstrates that HIS22 mainly reacts with population I ($IgM^{dull}IgD^{bright}$) cells; population III ($IgM^{bright}IgD^{dull}$) cells and a minority of population I express only very low levels of the HIS22 determinant. Spleen cell suspensions were stained with fluoresceinated anti-rat IgM (HIS40), biotinylated anti-rat IgD (MARD 3) and APhC-HIS22. Biotin was revealed by Texas red-avidin. (b) In spleen sections, HIS22 reacts with B cells in lymphoid follicles (F); B cells located in the MZ are virtually unstained. Immunoperoxidase staining of a frozen section taken from a rat spleen. Note that in this follicle no GC is present (animals were not deliberately immunized; × 125).

Furthermore, the $sIgM^-sIgD^-$ cells in the BM, which include B cell progenitors (pre-B and pro-B cells) do not express the determinant recognized by HIS22. Thus, this determinant appears relatively by late during B cell development, and is expressed in relatively high amounts on mature B cells, mainly found among population I ($IgM^{dull}IgD^{bright}$) cells.

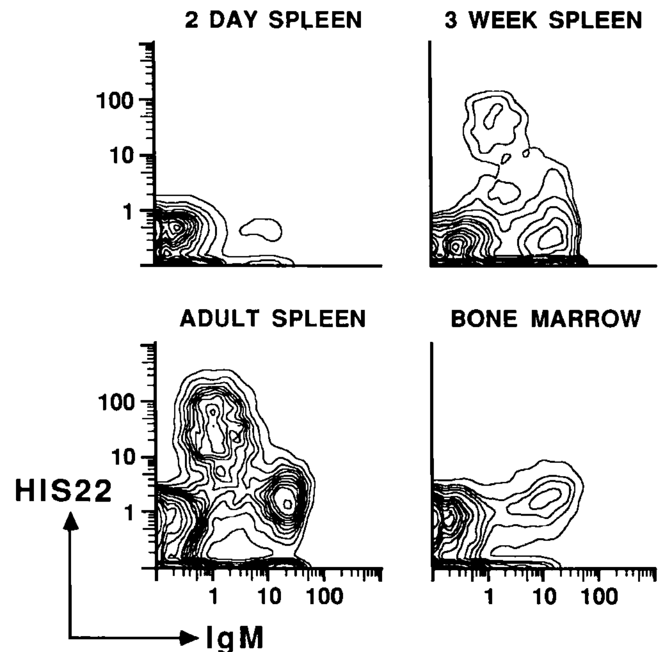


Figure 3. Two-color staining patterns of anti-rat IgM and HIS22 with rat spleen and BM. The majority of B cells in the BM and neonatal spleen express only low levels of HIS22 determinant. In adult spleen, most IgM^{dull} cells (population I, see Fig. 2) are brightly stained with HIS22. Cell suspensions were stained with fluoresceinated anti-IgM (HIS40) and APhC-HIS22.

3.2.3 HIS24 subdivides population III into two subpopulations

Fig. 4 shows that the vast majority of the splenic population I cells ($IgM^{dull}IgD^{bright}$) are bright for HIS24; however, population III cells ($IgM^{bright}IgD^{dull}$) are subdivided into two subpopulations. About one third of the cells within this

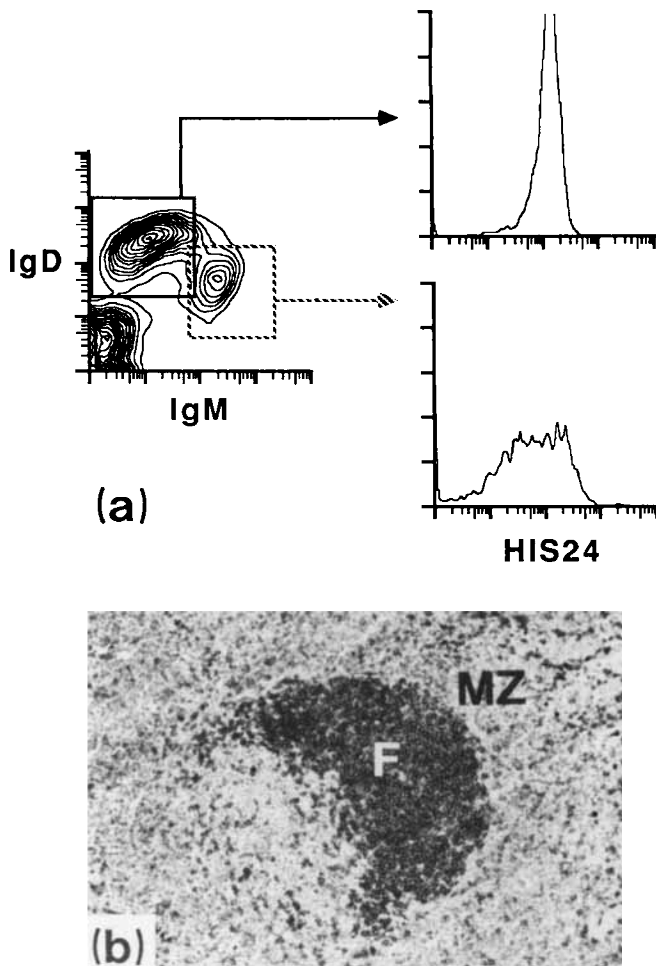


Figure 4. (a) Three-color immunofluorescence staining showing that HIS24 reacts brightly with virtually all poulation I ($IgM^{dull}IgD^{bright}$) cells and a small proportion of population III ($IgM^{bright}IgD^{dull}$) cells. About two thirds of population III cells express relatively low levels of the HIS24 determinant. Spleen cell suspensions were stained with FITC-anti-rat IgM (HIS40), biotinylated anti-rat IgD (MARD 3) and APhC-HIS24. Biotin was revealed by Texas red-avidin. (b) In spleen sections B cells expressing high levels of the HIS24 determinant are mainly found in lymphoid follicles (F) but not in MZ. Frozen section of rat spleen stained by immunoperoxidase with HIS24. Note that no GC is seen in this follicle. (× 125).

Table 2. HIS22 and HIS24 further subdivide population I and population III in adult rat spleen[a)]

	HIS14		HIS22		HIS24	
	bright	dull	bright	dull	bright	dull
$IgM^{dull}IgD^{bright}$ (population I)	99[b)]	1	64	36	96	4
$IgM^{bright}IgD^{dull}$ (population III)	98	2	13	87	38	62

a) Three-color FCM analysis of adult rat spleen cell suspensions stained with FITC-anti-IgM, biotin-anti-IgD (followed by Texas red-avidin) and APhC-conjugated HIS mAb.
b) Proportion of population I or population III cells; values express the mean of at least four different spleens.

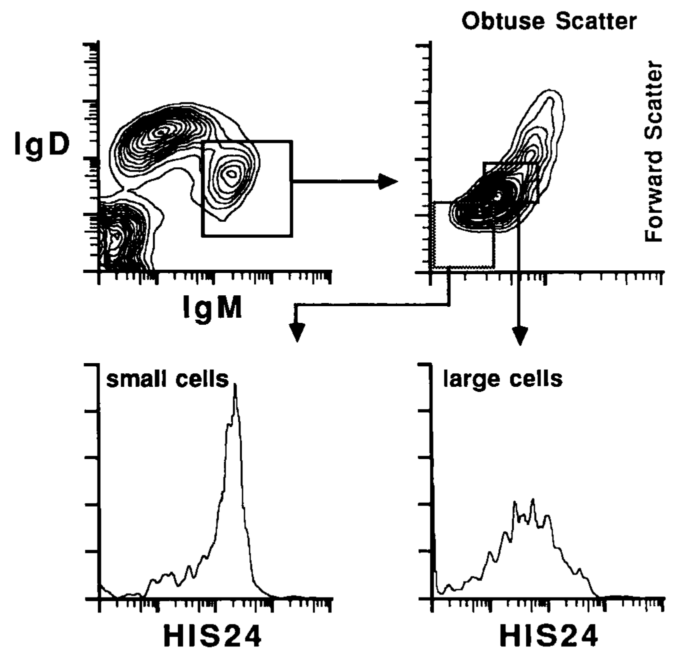


Figure 5. Three-color FCM analysis of rat spleen showing that relatively large population III cells ($IgM^{bright}IgD^{dull}$) are dull for HIS24, whereas the relatively small population III cells are bright for HIS24. Spleen cell suspensions were stained with fluoresceinated anti-rat IgM (HIS40), biotinylated anti-rat IgD (MARD 3) and APhC-HIS24. Texas red-avidin was used to reveal anti rat IgD.

subpopulation express relatively high amounts of HIS24 antigen, and roughly two thirds of the cells express relatively low amounts (about 5 times less than the $HIS24^{bright}$ cells; Table 2). The $HIS24^{dull}$ and $HIS24^{bright}$ cells are not well resolved, making accurate quantitation of either subset difficult. These two subpopulations identified with HIS24 tend to have distinctive sizes as measured by forward and obtuse scatter, *i.e.* the $HIS24^{bright}$ population III cells are relatively small cells, and the $HIS24^{dull}$ cells are relatively large cells (Fig. 5).

In previous studies, Opstelten et al. [14] have shown that the determinant recognized by HIS24 is expressed on early B cell progenitors in the BM, including terminal transferase-containing cells and μ chain-containing pre-B cells. Our FCM analysis indeed show that a large proportion of the $sIgM^-$ cells (which include all pro-B and pre-B cells) in the BM are $HIS24^{dull/bright}$ (Fig. 6). Virtually all $sIgM^+$ cells in rat BM are population III cells (Fig. 1), and these are all $HIS24^{bright}$ (Fig. 6). The $HIS24^{bright}$ population III cells also constitute the main B cell type found in spleens from neonatal (2-day- to 3-week-old) rats (Fig. 6) and are present in low numbers in adult rat spleen. By contrast the $HIS24^{dull}$ population III cells are almost absent from the BM and from neonatal (2–10-day-old) spleens. Thus, the HIS24 determinant is expressed in high amounts on the vast majority of population I cells and subdivides population III into two subpopulations. One expresses high levels of HIS24 determinant and is predominantly found on population III cells in the BM and in neonatal spleen and on a small subpopulation of population III cells from adult spleen. The second expresses only low amounts of HIS24

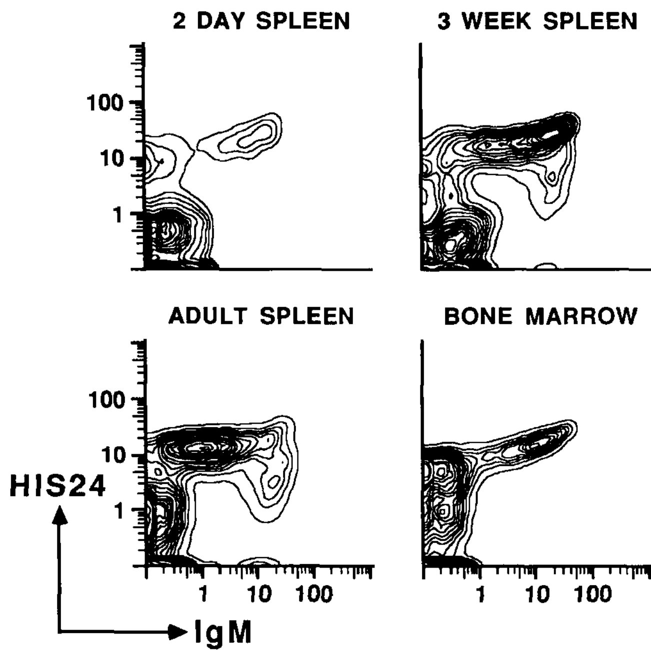
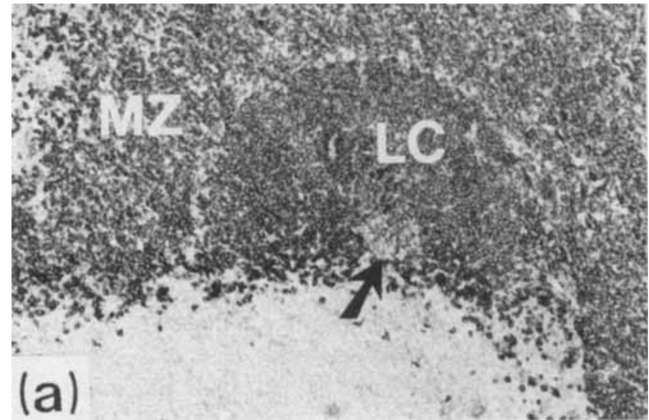


Figure 6. Two-color staining patterns with anti-rat IgM and HIS24, demonstrating that in neonatal rat spleen and in adult BM the vast majority of IgM^{bright} cells (population III) express high levels of the HIS24 determinant; in adult spleen a subpopulation (about two thirds) of the IgM^{bright} cells (population III, see Fig. 4) expresses relatively little HIS24 determinant. Cell suspensions were stained with FITC-anti-IgM (HIS40) and APhC-HIS24.



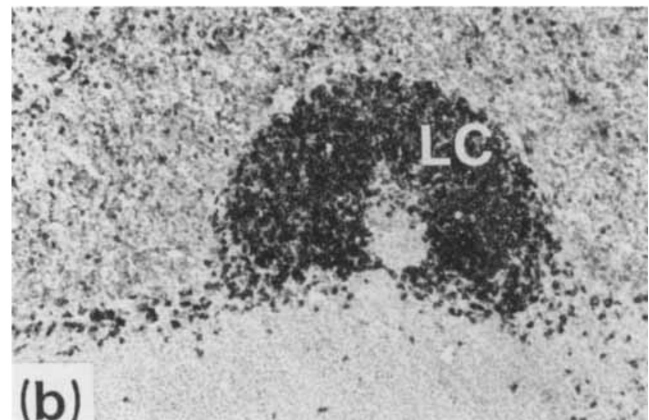


Figure 7. Reactivity patterns of serial sections from rat spleen with anti-IgM (a) and anti-IgD (b) showing that cells located in the lymphocyte corona (LC) of the lymphoid follicles express IgM and IgD, whereas MZ B cells express IgM but only very low levels of IgD. Note the presence of a very small (IgD^-) GC (arrow) in the follicle. Frozen sections of rat spleen (same animal as in Figs. 2 and 4) were stained by immunoperoxidase using anti-rat IgM (HIS40) or anti-rat IgD (MARD 3). (× 125).

determinant, contains relative large cells and is mainly found in adult spleens.

3.2.4 Follicular B cells are population I cells and MZ cells are population III cells

In the spleen, B cells are predominantly found in the white pulp, where they are localized in two distinct compartments, the lymphocyte follicle (after antigenic stimulation subdivided into GC and lymphocyte corona) and the MZ. Imunohistological staining with anti-IgM and anti-IgD antibodies demonstrate that MZ cells are mainly IgM^+IgD^- cells, in contrast with the follicular B cells that are IgM^+IgD^+ cells (Fig. 7; [6, 7]). Data presented here show that in adult rat spleen the HIS22 determinant is predominantly expressed by mature B cells, found mainly among the population I cells ($IgM^{dull}IgD^{bright}$). The HIS24 determinant is also expressed by the vast majority of population I cells, but in addition also reacts with a minor subset of population III cells. Figs. 2 and 4 show that in immunoperoxidase staining of frozen sections from adult rat spleen both the $HIS22^{bright}$ and the $HIS24^{bright}$ cells are predominantly located in the lymphoid follicles whereas MZ are virtually unstained with both antibodies. These immunohistological data are consistent with previous studies using different rat strains [10]. Thus, the immunohistologically defined phenotype of follicular B cells, *i.e.* $IgM^+IgD^+HIS22^+HIS24^+$, is similar to that of the predominant type of population I cells identified by multiparameter FCM analysis. In tissue sections MZ cells are $IgM^+IgD^-HIS22^-HIS24^-$ and are slightly larger cells than the follicular B cells. Thus these MZ cells are likely represented by the $HIS24^{dull}$ population III cells defined by FCM. We do not know where the $HIS24^{bright}$ population III cells are localized in tissue sections.

4 Discussion

Multiparameter (three-color) FCM analyses demonstrate a major phenotypic heterogeneity of rat B cells after staining for sIgM and sIgD in combination with a panel of mAb (HIS14, HIS22 and HIS24) directed to B cell-associated forms of the leukocyte common antigen (CD45R). These antibodies have previously been shown to react differentially with B cell subcompartments that can be distinguished in tissue sections of peripheral lymphoid organs [10], and prompted us to correlate here the FCM-defined B cell subpopulations with their corresponding anatomical counterparts.

Two-color FCM analyses for IgM/IgD of rat peripheral organs define the existence of at least two major subpopulations of B cells, similar to those previously defined in the mouse [1–3]. The predominant B cell population in adult rat

peripheral lymphoid organs (spleen, LN and Peyer's patches) expresses relatively low levels of sIgM and high levels of sIgD (population I); in the spleen but not in other peripheral lymphoid organs a substantial number of B cells express high levels of sIgM and relatively low levels of sIgD (population III). Population III cells, however, predominate in neonatal spleen, as in newborn mice [15–17]. The two IgM/IgD-defined subpopulations of adult rat spleen most probably correspond to the IgM^+IgD^+ and IgM^+IgD^- B cells that have been identified previously in rat spleen cell suspensions using a double-rosette assay [7]. Both their data and the data presented here show that in rats the proportion of splenic $IgM^{bright}IgD^{dull}$ cells is higher compared to mice; viz. by FCM, roughly 25% of the splenic B cells in rats are $IgM^{bright}IgD^{dull}$ cells whereas in BALB/c mice this proportion is approximately 10% [1].

A further heterogeneity of rat B cells is seen with respect to the expression of determinants recognized by the HIS22 and HIS24 mAb (but not with the pan-B cell antibody HIS14). The majority of population I cells ($IgM^{dull}IgD^{bright}$) present in peripheral lymphoid organs from adult animals, and thus the majority of peripheral B cells, express high levels of the determinants recognized by HIS22 and HIS24; only a small subset of population I cells do not express high levels of HIS22 antigen (but are $HIS24^{bright}$). In tissue sections cells with the predominant population I phenotype ($IgM^{dull}IgD^{bright}$ $HIS22^{bright}HIS24^{bright}$) are mainly located in lymphoid follicles (primary follicles and lymphocyte corona of secondary follicles; [6, 7, 10]). These B cells represent the recirculating pool of small resting B cells. A small subset of population I cells does not express high levels of HIS22 antigen (but are $HIS24^{bright}$). Their anatomical location is not clear, but their phenotype indicates that they are neither GC B cells nor MZ B cells. These cells might, however, also be present in primary follicles and the lymphocyte corona of secondary follicles, where some cells react only weakly with HIS22 (see Fig. 2).

Population III cells ($IgM^{bright}IgD^{dull}$) are almost exclusively found in the spleen and are essentially $HIS22^{dull}$. However, with HIS24 two subsets of population III cells can be distinguished: the major subset is intermediate in size (by forward scatter) and expresses relatively low levels of the HIS24 determinant. The second subset consists of small lymphocytes which express relatively high levels of the HIS24 determinant. In tissue sections B cells located in the splenic MZ are intermediate-size cells and stain intensely with anti-IgM, but only weakly with anti-IgD, HIS22 and HIS24 [6, 7, 10]. Thus, among population III cells identified by FCM only the $HIS24^{dull}$ B cells can be MZ B cells. This observed heterogeneity of population III cells has important implications: it clearly indicates that for studies of MZ B cells in cell suspensions the use of IgM and IgD alone is not sufficient to define these cells and may for example lead to an overestimation of the proportion of MZ B cells and of course to erroneous conclusions about capabilities of $sIgD^{dull}$ B cells in functional assays [18, 19].

We consider the $HIS24^{bright}$ population III cells as relative immature B cells that have recently been derived from the BM. First, these cells are the predominant $sIgM^+$ B cells found in the BM. Second, it is the major B cell population found in neonatal spleen cell suspensions and the decrease of the proportion of these cells during ontogeny coincides with the observed increase in the proportion of population I cells (mature resting B cells). Moreover, during the first week of life, when these $HIS24^{bright}$ population III cells predominate, follicles are not observed [20]. Consistent with the idea that $HIS24^{bright}$ population III cells constitute the newly formed B cell population are findings by Bazin et al. [21] and MacLennan and Gray [22], who showed that after cessation of treatment of rats with anti-IgM and anti-IgD antibodies to suppress B cell development, the first B cells that appear in the spleen express sIgM but not sIgD and are $HIS24^{bright}$. Similarly, in the mouse immature, newly formed B cells, identified by the simultaneous expression of the antigens BLA-1 and BLA-2, are also included within population III of the spleen [3]. The virtual absence of $HIS24^{bright}$ population III cells in LN and Peyer's patches indicates that B cells recently formed in the BM selectively (?) enter the spleen where they may differentiate further to mature recirculating follicular B cells (and possibly also to splenic MZ B cells). Indeed, after neonatal or adult splenectomy there is an accumulation of relative immature cells in LN suggesting an active role of the spleen in B cell differentiation [23]. One such role of the spleen could be the proposed (positive) selection of newly formed B cells, just derived from the BM, by virtue of their interaction with T cells and antigen presented on the surface of dendritic cells [22]. It remains formally possible that these $HIS24^{bright}$ population III cell represent a distinct class of B cells rather than a differentiation stage of B cell development. We do not know exactly where the $HIS24^{bright}$ (and $IgM^{bright}IgD^{dull}HIS22^{dull}$) population III cells are located in tissue sections of the spleen: the phenotype of these cells does not fit with that of MZ B cells or follicular B cell (either lymphocyte corona or GC). However, as

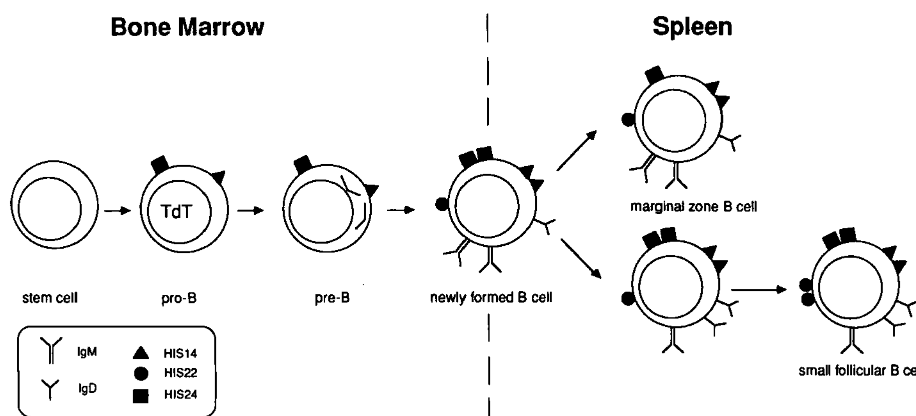


Figure 8. Model of B cell differentiation pathways in the rat. Newly formed B cells are thought to act as a common precursor cell for both MZ B cells and follicular B cells (*i.e.* B cells in the lymphocyte corona). Low *vs.* high expression of a determinant is indicated by one or two icons for that determinant, respectively.

stated above, HIS24^{bright} cells are mainly located in lymphoid follicles and it could therefore be possible that these HIS24^{bright} population III cells are located in follicles (lymphocyte corona) as well.

The possible lineage relationship(s) between different subpopulations in rat spleen is not precisely known. MacLennan and co-workers speculate that follicular B cells (which we show are contained within population I) and MZ B cells (*i.e.* the HIS24^{dull} population III cells) develop as separate lineages [5, 22]. This is based upon findings that treatment of rats from birth with anti-IgD antibodies results in the development of a spleen in which MZ are present whereas follicles (*viz.* primary follicles and lymphocyte coronas) are lacking [6]. Furthermore, follicular B cells can develop in the absence of MZ B cells: after total body irradiation of rats with hind limb shielding the regeneration of follicles precedes that of the splenic MZ [24]. In the mouse, two B cell lineages are distinguished based upon the (loss of) expression of the antigens BLA-1 and BLA-2 [3]. Accordingly, relatively immature B cells, expressing both BLA-1 and BLA-2, may give rise separately to a predominant population of double-negative population I cells and to (BLA-2^+) population III cells.

These findings in rats and mice, together with the observed phenotypical heterogeneity of rat peripheral B cells as described in this study, led us to propose a model of B cell differentiation in the rat as is shown in Fig. 8. We would like to stress here that in addition to these BM-dependent B cell differentation pathways, also other BM-independent B cell lineages (see *e.g.* [25]), like the murine Ly-1 B cell lineage, could be present in the rat. However, we could not yet unequivocally establish the presence of such CD5 B cells in the rat. From our data it follows that the HIS24 determinant appears early in B cell development and the HIS22 determinant late (see also [14]). In this model the population of relatively immature B cells (*i.e.* the HIS24^{bright} population III cells) is thought to act as a common precursor that can differentiate either to MZ B cells or through an intermediate population of HIS22^{dull} population I cells to the predominant mature B cell population (the follicular B cells proper). Transfer studies with sorted populations of B cells in combination with congenic pairs of animals are required to evaluate this model and to test relationships of phenotypically defined B cell subpopulations.

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