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# Development of B-cell subsets

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## INTRODUCTION

Antibody production is the key factor in the humoral defense against invading pathogens and other potentially harmful substances that may be introduced into the body. Antibody-forming cells (AFC) are derived from specialized lymphocytes named B cells, which are individually differentiated to produce antibody molecules that have distinctive antigen-combining sites (specificities) and functional components (isotypes).

The enormous diversity of antibody reactivities required to protect against the wide variety of antigens that an animal may encounter throughout life is generated by a complex differentiation process consisting of two basic stages. The variable region, which defines the antigen-combining site of the unique immunoglobulin molecule

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that each B cell produces, is initially determined by the developmentally-controlled juxtaposition of $V_H$, D and $J_H$ elements of the heavy chain and $V_L$ and $J_L$ elements of the light chain. Variable-region rearrangement occurs during the early stages of B cell differentiation in the bone marrow and fetal liver. The first constant region domains (μ and δ) used in B cell development are determined by differential processing of the RNA transcripts. Later, as mature B cells in the periphery encounter specific antigens, the antibody molecules they produce may be further diversified by mechanisms that enable 'switching' of constant region (isotype) concomitant with somatic mutation of variable-region genes. This latter process, which can ultimately yield antibodies with higher affinities for the antigen, matures from a series of cell clones producing distinct but related immunoglobulin (Ig) molecules that often have markedly different binding affinities for the antigen.

The differentiation stages through which B cells and their progenitors pass on their way to becoming AFC initially appeared to follow a single developmental pathway. However, phenotypic and cell transfer studies begun in the early 1980s identified at least two independent B cell development pathways (lineages) whose progenitors are now distinguishable at an early developmental stage (pro-B cell), when the first Ig ($D–J_H$) rearrangement begins (Hayakawa *et al.*, 1984, 1985). T cells have now also been shown to differentiate along similarly separable developmental lineages, suggesting that the commitment to give rise to particular T and B lineages reflects the activity of distinctive lymphoid stem cells (Ikuta *et al.*, 1990).

In this chapter, we focus primarily on the features that distinguish the developmental pathways of the two most well-established B cell lineages, commonly referred to as the B-1a (earlier designated Ly-1 or $CD5^+$ B) and conventional (or B-2) lineages (Kantor *et al.*, 1991). We begin with a relatively brief description of the phenotypic and functional characteristics of the developmental stages of B cells. Then, starting with the most mature B cells and working back towards the early stages of B cell development, we detail the differences between the cells in each lineage at these various stages of development. Finally, we discuss the features that potentially distinguish additional B cell developmental lineages and discuss the evolutionary and functional consequences of these multiple layers of functionally related cells in the immune system.

## FEATURES OF B-CELL DEVELOPMENT

The progressive Ig gene rearrangements that occur as cells move along the B cell developmental pathway are accompanied by differentiation events that alter the size and surface phenotype of the developing cells (summarized in Table I). This process has been well studied for conventional B cell development in adult bone marrow (Hardy *et al.*, 1991; Rolink and Melchers, 1991). In essence, the initial Ig heavy chain rearrangement, which joins a D and a $J_H$ element is accompanied by a shift from the pro-B cell to the early pre-B phenotype. The rearrangement that joins this



**Table I** Phenotypic changes during B-cell development

| | Pro-B cells | Pre-B cells | Immature B cells | Mature B cells | Memory B cells | AFC plasma cells |
|---|---|---|---|---|---|---|
| Surface Phenotype | | | | | | |
| IgM | Negative | Negative | High | Intermediate | Negative | High* |
| IgD | Negative | Negative | Neg/low | High | Negative | Low* |
| B220 | Low | Intermediate | High | High | ? | Low* |
| HSA | Negative | Intermediate | High | Low | ? | High*† |
| CD43 | High | Low | Low | Negative | ? | High |
| CD23 | Negative | Negative | Negative | High | ? | Negative* |
| BP-1 | Negative | High | Negative | Negative | ? | ? |
| Size (FSC) | Large | Large/small | Small | Small | Small | Large*† |
| Ig gene rearrangements | Heavy chain D to J | Heavy chain V to DJ light chain | Rearranged | Rearranged | Rearranged/isotype switching | Rearranged/isotype switching |
| Functional | Antigen unresponsive | Antigen unresponsive | Selection/ tolerance? | Antigen responsive | Late stages of response | Late stages of response |
| Role of antigen | Antigen independent | Antigen independent | Antigen independent | Antigen dependent | Antigen dependent | Antigen dependent |
| Anatomic sites | Bone marrow | Bone marrow | Bone marrow and periphery | Bone marrow and periphery | Periphery | Periphery |

* Antigen-specific activation of B-1 cells *in vivo*.
† Activation of spleen cells *in vitro*.
These data are based upon the following references: Allman, *et al.*, (1993); Hardy *et al.*, (1984, 1991); Hathcock *et al.* (1992); Metcalf and Klinman, 1977; Nishimura *et al.* (1992); Nossal, (1983); Rolink and Melchers (1991); Waldschmidt *et al.*, (1992).

D-$J_H$ segment to a variable-region ($V_H$) gene is accompanied by a shift to the late pre-B stage. Transcription and splicing of the RNA creates an expressible μ heavy chain that is found in the cytoplasm and probably on the surface in association with the pseudo-light chain proteins, λ5 and $V_{pre\text{-}B}$ (Rolink and Melchers, 1991). The rearrangement of the light chain and the expression as surface IgM move the cell to the immature B cell stage. Finally, B cells migrate to the periphery where they are selected into the long-lived pool of recirculating B cells. This is marked by an increase in IgD expression and additional phenotypic changes that define the cell as a mature conventional B cell.

Hardy and Hayakawa have defined distinctive FACS (Fluorescence Activated Cell Sorter) phenotypes of the pro-B and pre-B cell developmental stages (see Table I) (Hardy *et al.*, 1991). These differences primarily involve coordinated quantitative changes in the expression of several surface molecules, notably leukosialin (CD43/S7), B220/6B2, and heat stable antigen (HSA; 30-F11), as well as the IgM molecule itself. As conventional B cells are selected into the mature pool, they can be distinguished by surface expression of IgM, IgD, CD23 and low levels of HSA. Following antigen-specific activation, the mature B cells can undergo additional differentiation steps to become AFC/plasma cells or memory B cells. Plasma cells are the differentiated effector cells responsible for producing large amounts of secreted Ig, while memory B cells, which persist for long periods and can later give rise to plasma cells, are crucial for the immune system's ability to mount an accelerated and enhanced response upon its second encounter with a particular antigen (Gray, 1993; Parker, 1993).

## B-CELL SUBSETS AND LINEAGES

Early studies of lymphocyte subsets focused largely on the T cell compartment. Antibody-mediated cytotoxicity and FACS studies showed that expression of the Lyt-2 (CD8) surface molecule distinguishes the suppressor/cytotoxic subset from the helper/inducer subset (Cantor and Boyse, 1975, 1977). The expression of Lyt-1 (CD5) was initially used in antibody-mediated cytotoxicity studies to identify and deplete the helper/inducer subset (Herzenberg *et al.*, 1976); however, subsequent FACS studies with monoclonal anti-CD5 antibodies showed that the difference in CD5 expression between the subsets was quantitative rather than qualitative (Ledbetter *et al.*, 1980). The use of anti-CD5 in T cell subset studies terminated with discovery of the CD4 surface antigen, whose expression on the helper/inducer subset definitively distinguishes this subset. Ironically, the expression of CD5 later proved to distinguish the B cell subsets/lineages (Lanier *et al.*, 1981; Hardy *et al.*, 1982; Manohar *et al.*, 1982; Herzenberg *et al.*, 1986).

B cells were initially thought to be much more homogeneous than T cells, differing principally with respect to the isotype and specificity of the Ig molecules they produced. Phenotypically distinct subsets of B cells were recognized; however, these subsets tended to be viewed as consisting of B cells at different stages of differentiation (e.g. naive and memory B cells) that have linearly descended or alternatively differentiated from the same progenitors to provide specialized capabilities related to antigen recognition or antibody production.



This paradigm was replaced/extended by the recognition that B cell progenitors are committed to differentiate along developmental pathways that culminate in the development of different functional subsets. By now, a wide variety of phenotypic, anatomical, developmental and functional differences between these major B cell lineages have been verified. Two major lineages are generally recognized: conventional B cells (also called B-2 cells), which develop predominantly from progenitors in the adult bone marrow; and B-1a cells, which develop predominantly from fetal progenitors (Kantor and Herzenberg, 1993). The genetically defined developmental program of these lineages is crucial to defining their overall capabilities and has an important, possibly decisive, influence in defining their basic antibody repertoires and the specific roles they play in immune functions.

Phenotypic, locational and functional differences between the two major B cell lineages can be found at every stage of development; however, considerably more is known about these differences amongst the mature B cells. Thus, we begin here by defining the developmental and functional subsets of mature B cells and their progeny within each lineage and contrasting the lineage to lineage variation of these mature subsets.

## DISTRIBUTION AND PHENOTYPIC CHARACTERISTICS OF B CELLS IN PERIPHERAL LYMPHOID ORGANS

B cells are small lymphocytes that express surface Ig molecules. In general, they do not secrete Ig but can be stimulated to differentiate into phenotypically distinct AFC responsible for performing this function. In adult animals, the majority of all B cells located in the peripheral lymphoid organs (i.e. spleen, lymph nodes and Peyer's patches) belong to the conventional B cell lineage. Small numbers of B-1 lineage cells can be found in these organs, most notably in spleen; however, most studies characterizing B-1 cells draw these cells from the peritoneal cavity, where they represent the predominant B cell population (Hayakawa *et al.*, 1985; Herzenberg *et al.*, 1986; Kantor and Herzenberg, 1993).

### Conventional B cells

The major conventional B cell subset (follicular B cells) contains cells that are relatively small and found predominantly in lymphoid follicles, where they express low levels of IgM, high levels of IgD and characteristic levels of other surface molecules (Table II, Figs 1 and 2). These B cells constitute virtually the entire B cell population in lymph nodes; however, in the spleen, the overall B cell population also

**Table II** Phenotype of peripheral B-cell subsets

| | Follicular | Immature | Marginal zone | B-1 |
|---|---|---|---|---|
| IgM | Intermediate | High | High | High |
| IgD | High | Negative/low | Low | Low |
| CD45 | High | High | High | High |
| B220 (6B2) | High | High | Intermediate | Low |
| B220 (2C2) | High | High | High | High |
| HSA (J11d) | Intermediate | ? | High | Intermediate |
| HSA (M1/69) | Low | High | Intermediate | Intermediate |
| HSA (53–10) | Negative | ? | Intermediate | Intermediate |
| CD23 | High | Negative | Negative | Negative |
| CD22 | High | Low | High | High |
| L-selectin (MEL-14) | High | Low ? | Low | Negative |
| CTLA-4Ig-binding | Negative | Negative | Negative | Low |
| CD43 (S7) | Negative | Low | Negative | Intermediate |
| MAC-1 (CD11b) | Negative | Negative | Negative | Spleen: negative |
| CD5 | Negative | Negative | Negative | PerC: low<br>B-1a: low<br>B-1b: negative |
| Size | Small | Small | Small | Large |

The values given here represent the relative intensity of the major population within each subset. For any given antigen there can be considerable heterogeneity within the subset. For more detailed analysis see the FACS plots in Fig. 2. The data are based upon the following references: Manohar *et al.* (1982); Hayakawa *et al.* (1983); Hardy *et al.* (1984); Herzenberg *et al.* (1986); Kantor *et al.* (1992); Nishimura *et al.* (1992); Waldschmidt *et al.* (1992); Allman *et al.* (1993); Wells *et al.* (1994).

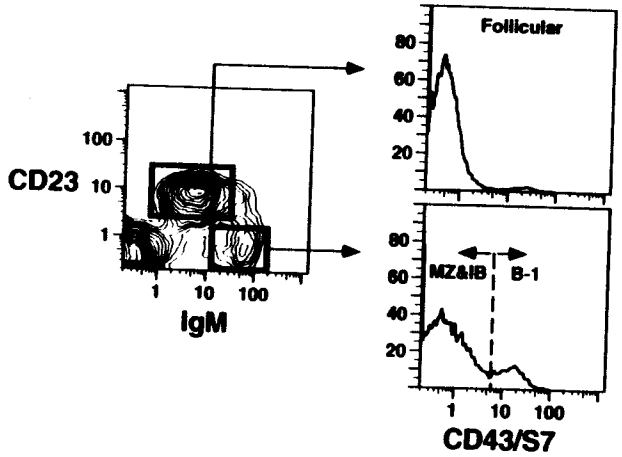


**Fig. 1.** Phenotypic identification of splenic B cell subpopulations. Spleen cells from 1–2-month-old BAB/25 mice were stained for CD23 (TexasRed [TR]), IgM (Fluorescein [FI]) and CD43/S7 (Phycoerythin [PE]). The CD23, IgM phenotype of total spleen cells is shown in the left-hand panel. Both marginal zone and immature cells (MZ&IB) and B-1 cells are found within the $IgM^{bright}$, $CD23^{-}$ gate, but these two populations can be distinguished by their CD43/S7 phenotype. The MZ&IB, which make up the majority of this population, are $IgM^{bright}$, $CD23^{-}$, $CD43/S7^{-}$. The B-1 cells, however, are $IgM^{bright}$, CD23 , $CD43/S7^{+}$.



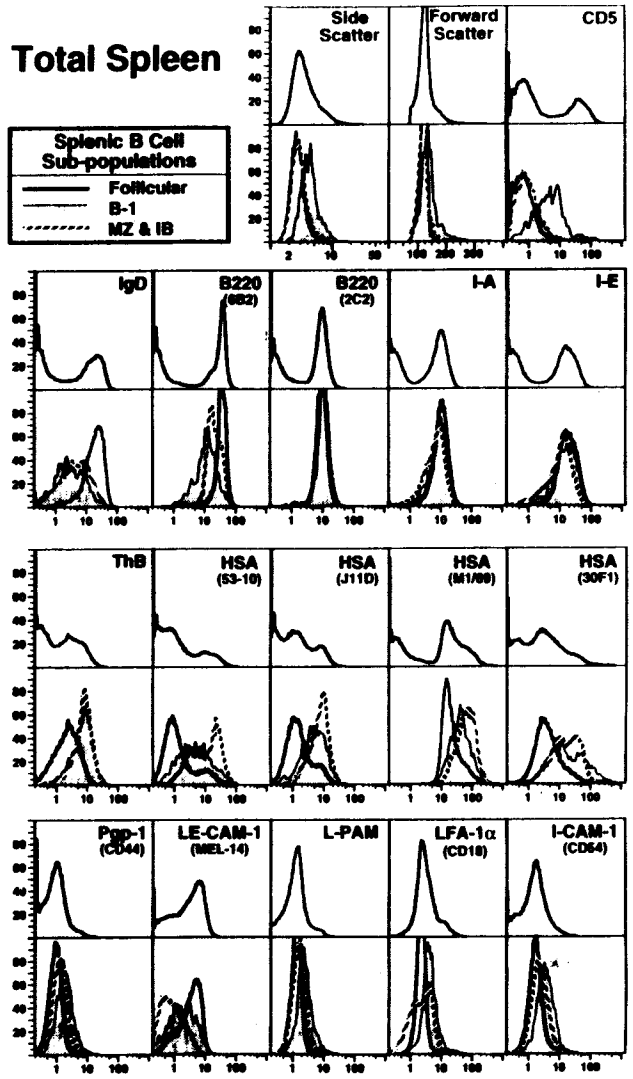


**Fig. 2.** Comparative phenotypes of splenic B-1 and marginal zone and immature B cells (MZ&IB). Spleen cells from 1–2-month-old BAB/25 mice were stained for CD23 (biotin [bi]), IgM (Fluorescein [FI]), CD43/S7 (Phycoerythin [PE]) and CD5 (allo phycocyanin [APC]). In all other samples, cells were stained for CD23 (TexasRed [TR]), IgM (APC), CD43/S7 (PE) and FI conjugates of antibodies to the antigens specified. The expression of each antigen on total spleen (upper histograms) and on follicular, B-1 cells and MZ&IB cells (lower histograms) is shown. The B cell subpopulations were gated as shown in Fig. 1. The *y*-axis is a normalized scale for each histogram. Follicular, MZ&IB and B-1 cells comprise approximately 85%, 12% and 3% of the total B cells respectively. All antibodies were obtained from Pharmingen (San Diego) with the exception of anti-IgM (331) and anti-ThB (49H4) which were prepared in our laboratory.

contains marginal zone (MZ) B cells, which constitute a small, clearly distinguishable subset (roughly 5–10% of total splenic B cells) that expresses higher levels of surface IgM, less IgD, more HSA and does not express CD23 (see Table I) (Kroese *et al.*, 1990a; Waldschmidt *et al.*, 1992). As their name implies, MZ B cells are found in areas called marginal zones, which surround the follicles. Thus the MZ subset is phenotypically, anatomically and functionally distinct from the follicular B cell subset.

Adult spleen also has a small number of immature conventional B cells. Although the phenotype of these cells, which are most likely recent immigrants from the bone marrow, readily distinguished them from follicular B cells, they were not initially distinguishable from either B-1 or MZ B cells. Cancro and colleagues solved this problem by demonstrating that, like immature B cells in the bone marrow, these putative recent immigrants express distinctively high levels of HSA (M1/69) (Waldschmidt *et al.*, 1992; Allman *et al.*, 1993; Wells *et al.*, 1994). In addition, Waldschmidt and colleagues have recently shown that immature B cells express lower surface levels of CD22 than mature cells in the spleen, including both the follicular and MZ B cell subsets (T. Waldschmidt, personal communication).

### B-1 cells

Unlike conventional B cells, B-1 cells are seldom found in lymph nodes and Peyer's patches and represent only 1–3% of the total B cells in the adult spleen (Herzenberg *et al.*, 1986; Kantor *et al.*, 1992; Kantor and Herzenberg, 1993; Wells *et al.*, 1994). Anatomically, B-1 cells are not concentrated in one particular area of the spleen and tend to be found most frequently in the red pulp, occasionally in the follicles, and rarely in marginal zones (Kroese *et al.*, 1990b, 1992). Phenotypically, the B-1 population in the spleen, as in the peritoneal cavity (see below), is similar to MZ B cells with respect to expression of IgM, IgD and HSA and the failure to express CD23. However, B-1 cells typically express CD43 (leukosialin) whereas neither follicular nor MZ B cells express this antigen. Furthermore, they tend to be larger in size than most MZ and conventional B cells (Wells *et al.*, 1994).

Although B-1 cells are rare in spleen and lymph nodes, they represent the predominant B cell population in the peritoneal and pleural cavities and are most readily characterized when taken from this source. In a BALB/c mouse, B cells typically represent more than 80% of the roughly $7–8 \times 10^6$ lymphocytes recoverable from the peritoneal cavity. B-1 cells typically comprise 60–80% of these B cells (depending upon strain). Conventional B cells that are phenotypically identical to the follicular B cells found in the spleen and lymph nodes comprise the remainder (Herzenberg *et al.*, 1986; Kantor and Herzenberg, 1993).

Peritoneal and splenic B-1 cells are phenotypically identical with one major exception: splenic B-1 cells do not express MAC-1 (CD11b), whereas peritoneal and pleural B-1 cells express detectable levels of this surface antigen. Curiously, this difference in MAC-1 expression appears either to be environmentally determined or to determine the migratory behavior of the B-1 cells on which it is expressed: FACS-sorted splenic B-1 cells, which lack MAC-1, reconstitute peritoneal B-1 cells that express MAC-1 in transfer recipients; and, conversely, peritoneal B-1 cells that express MAC-1 reconstitute splenic B-1 cells that do not express this antigen (Kantor *et al.*, 1995).



B-1 cells were originally called Ly-1 B cells. This earlier name reflected their initial recognition as a subset of B cells that expresses CD5, a pan T cell surface glycoprotein formerly called Lyt-1 or Ly-1 (see above). The current name (B-1) was provisionally selected to allow inclusion of another subset/lineage of cells that do not express CD5 but are otherwise phenotypically and locationally indistinguishable from the $CD5^+$ B-1 cells (Kantor *et al.*, 1991). In this terminology, which is now widely used, B-1 cells that express CD5 are referred to as B-1a cells and the B-1 cells that do not express CD5 are referred to as B-1b cells. Other differences between B-1a and B-1b cells include differences in antibody repertoire (Kantor *et al.*, 1994), in frequency of occurrence in different mouse strains (Herzenberg *et al.*, 1986; Stall *et al.*, 1992; Wells *et al.*, 1994), and in the time at which they develop (Kantor *et al.*, 1992; Stall *et al.*, 1992). These differences, particularly at the developmental level, suggest that B-1a and B-1b cells belong to distinct, albeit closely related lineages (see below).

The surface phenotypes of the adult splenic B cell subsets described are shown in details in Figs 1 and 2 and summarized in Table II. Follicular B cells from both spleen and lymph node are most easily identified as small lymphocytes that express CD23, low surface levels of IgM, high levels of IgD, and high levels of B220/6B2. MZ, immature and B-1 cells, in contrast, all express high levels of IgM, low levels of IgD and no CD23. These latter three populations, which are found in the spleen, are distinguished by their differential expression of HSA, B220/6B2, CD22 and CD43: immature B cells express high levels of HSA, intermediate B220/6B2, low CD22, and do not express CD43; MZ B cells express intermediate levels of HSA and B220/6B2, high CD22, and also lack CD43 expression; B-1a ($CD5^+$) and B-1b ($CD5^-$) cells in both the spleen and peritoneal cavity express intermediate levels of HSA, low B220/6B2, high CD22 and, in contrast to follicular, MZ and immature B cells, low levels of CD43. Finally, splenic B-1 cells do not express MAC-1 whereas peritoneal B-1 cells express low levels of MAC-1 (Kantor *et al.*, 1992; Waldschmidt *et al.*, 1992; Allman *et al.*, 1993; Wells *et al.*, 1994).

## SELF-REPLENISHMENT OF B-CELL SUBSETS

In addition to the unique phenotypic and anatomical characteristics of B-1 and conventional B cells, distinct functional differences further define these two lineages. A feedback mechanism regulates development of the B-1 population from immature progenitors. Decreasing B-1a progenitor activity plus this feedback regulation results in the inhibition of newly emerging B-1 cells into the periphery after weaning, while still allowing conventional B cells to continue to develop from Ig progenitors in the adult bone marrow (Lalor *et al.*, 1989a,b; Watanabe, L.A. Herzenberg and A.B.

Kantor, in preparation). Data demonstrating that B-1 and conventional B cells turn over at similar rates (Förster and Rajewsky, 1990; Deenen and Kroese, 1993), and that adoptively transferred peritoneal B-1 cells completely replenish the peripheral B-1 population (Hayakawa *et al.*, 1986) demonstrate that the B-1 lineage is mainly self-replenished from Ig$^+$ cells. In contrast, adoptive transfers of splenic or lymph node conventional B cells indicate that although these cells may persist for long periods, they have little or no capacity for expansion or self-replenishment (Sprent *et al.*, 1991; Kantor and Herzenberg, 1993; Kantor, 1995).

A number of experiments suggest that the preferential expansion/self-replenishment of the B-1 cells may be the result of ongoing receptor stimulation, possibly due to the low-affinity self-reactive specificities common within the population. Analysis of B-1 cell hyperplasias in NZB/W mice show that individual clones within the same mouse utilized the same $V_H$, $D_H$ and $V_L$ genes, indicating that the expansion was associated with a given specificity or idiotope (Tarlinton *et al.*, 1988). Similarly, the expansion of anti-phosphatidylcholine reactive B-1 cells, which account for 10–15% of peritoneal B-1, has been attributed to antigenic selection and expansion (Pennell *et al.*, 1988, 1989, 1990). This is supported by the fact that a large fraction of B-1 cells specific for phosphatidylcholine (bromelain-treated mouse erythrocytes) appear to have their membrane immunoglobulin (mIg) occupied by antigen (Carmack *et al.*, 1990). However, these data, which indicate antigenic stimulation, do not undermine the lineage distinction between B-1 and conventional B cells, which is based on developmental differences that are manifest very early in the B cell developmental pathway.

## DIFFERENTIATION OF B CELLS IN RESPONSE TO ANTIGENIC STIMULATION

Primary immune responses to both T-independent (TI) and T-dependent (TD) antigen are characterized by a relatively weak, short-lived burst of antibody (generally IgM) production. TD antigens are also able to elicit secondary or anamnestic responses resulting in a more rapid rise in antibody level and a significantly higher steady-state level that persists long after first contact with the antigen. The cells responsible for the antibody production in the secondary response have undergone affinity maturation in order to produce antibodies that are more effective in clearing the invading antigen.

Secondary responses are also characterized by a switch to the production of isotypes other than IgM. Each Ig heavy chain isotype exhibits a unique pattern of effector functions that participate in these host-defense responses. B-1 cells expressing isotypes other than IgM have been found *in vivo* (Herzenberg *et al.*, 1986; Förster and Rajewsky, 1987; Kroese *et al.*, 1989; Solvason *et al.*, 1991). Furthermore *in vitro* studies (Braun and King, 1989; Waldschmidt *et al.*, 1992; Tarlinton *et al.*, 1994; Whitmore *et al.*, 1992) have shown that isotype switching can occur in B-1 cells.



However, conventional B cells participating in antibody responses tend to switch to certain isotypes more frequently than do B-1 cells, resulting in quite distinct isotype profiles in antibody responses produced by these two lineages (Herzenberg *et al.*, 1986). The significance of this difference in isotype profiles between B-1 and conventional B cells is not yet known, but understanding these differences will be important in determining the role of B-1 cells in immune responses.

In addition to isotype switching, antigen-stimulated cells undergo affinity maturation, which is characterized by production of Ig whose combining site structure has changed due to somatic hypermutation. This somatic hypermutation process, which characteristically occurs during the generation of memory B cells, appears to occur substantially less frequently in B-1 cells than in conventional B cells. Analysis by Hardy and colleagues of B-1 cells that produce anti-phosphatidylcholine antibodies demonstrated that both the variable heavy chain ($V_H$) and variable light chain ($V_L$) remain totally unmutated (Hardy, 1989). Shirai *et al.* (1991) in contrast, have found evidence of somatic mutation in neoplastic B-1 cells. These findings suggest that B-1 and conventional B cells differ with respect to somatic mutation; however, data are too sparse at present to determine whether these differences reflect inherent differences in the ability to undergo somatic mutation or whether they stem secondarily from other differences between the two types of B cells, e.g. the local environment in which the antibody response develops; the basic antibody repertoire from which the response is drawn; or the nature of the T cell help evoked by the antigen and/or the responding B cell.

Generally, B cells give rise to two distinct cell types in primary responses to TD antigens: AFC or plasma cells that secrete predominantly low-affinity IgM antibodies and are located in the periphery of the periarteriolar lymphoid sheaths (PALs) of the spleen and lymph nodes (van Rooijen *et al.*, 1986); and memory B cells, which (according to current understanding) are generated in the germinal centers of peripheral lymphatic organs and upon later stimulation yield the plasma cells responsible for the high-affinity, predominantly non-IgM antibodies produced during secondary responses (Coico *et al.*, 1983; for review see Gray, 1993.

Several theories have been proposed to account for the simultaneous generation of these two populations. The most widely accepted explanation holds that an unequal division or differentiation of primary B cells following antigen-specific activation results in the formation of both AFC and memory B cells (Williamson *et al.*, 1976). In support of this, Jacob and Kelsoe (1992) have shown that the cells responsible for early antibody production in the primary response to (4-hydroxy-3-nitrophenyl)acetyl (NP) share a common clonal origin to germinal center B cells arising in the same response. Other studies by Klinman and colleagues, however, strongly suggest that there are two distinct populations of B cells responsible for the primary response (J11d$^{low}$) and secondary responses (J11d$^{hi}$) (Linton *et al.*, 1989, 1992; Linton and Klinman, 1992) These 'separate populations' and 'unequal division' theories, however, are not mutually exclusive. Properties of the antigenic response being studied (dose, route of injection, etc.) as well as the B cell subsets involved may favor one model over the other in a given situation.

## DIFFERENCES IN REPERTOIRE BETWEEN B-CELL SUBSETS

Unlike conventional B cells, the B-1 population has a repertoire that seems to be skewed toward the production of antibodies reactive with self antigens such as bromelain-treated mouse red blood cells and DNA (Hayakawa *et al.*, 1984; Mercolino *et al.*, 1986, 1988). B-1 cells also respond well to many TI antigens, particularly those associated with microorganismal coat antigens such as lipopolysaccharide (Su *et al.*, 1991), phosphorylcholine (PC) (Masmoudi *et al.*, 1990; Taki *et al.*, 1992), $\alpha(1\rightarrow3)$ dextran (Förster and Rajewsky, 1987) and $\alpha(1\rightarrow6)$ dextran (Wang *et al.*, 1994); however, they do not respond to all TI antigens and they do respond to certain T-dependent (TD) antigens (see below). Similarly, although conventional B cells usually produce the high-affinity (affinity-matured) memory responses to TD antigens, they also can produce low-affinity primary responses to certain TI antigens. Thus, although some general trends exist, the responses of B-1 and conventional B cell responses cannot be definitively categorized according to the nature of the responses in which they participate.

The unique repertoire and responsiveness of the B-1 population has generated considerable speculation on the idea that B-1 and conventional B cells can be distinguished by their ability to respond to TI and TD antigens, respectively. Indeed, the acquisition of the B-1 phenotype itself has been proposed to result from the interaction of a resting conventional B cell with a TI antigen (Rabin *et al.*, 1992; Haughton *et al.*, 1993). This theory, however, is inconsistent with data, alluded to above, showing that the responses of B-1 and conventional B cells cannot be categorized solely as TD or TI. For example, B-1 cells do not respond well to many TD antigens, including sheep erythrocytes, Trinitrophenol (TNP) and NP haptens, presented in TD forms (Förster and Rajewsky, 1987; Hayakawa and Hayakawa *et al.*, 1984; Hardy, 1988); however, they produce virtually the entire primary TD response to phosphorylcholine-keyhole limpet hemocyanin (PC-KLH) (which is composed of T15id$^+$ antibodies) (Masmoudi *et al.*, 1990; Taki *et al.*, 1992). Furthermore, although they do respond to certain TI antigens, B-1 cells do not respond at detectable levels to antigens such as TNP or NP presented in a TI form, i.e. coupled to ficoll (Hayakawa *et al.*, 1984; Förster and Rajewsky, 1987; Hayakawa and Hardy, 1988).

Data from studies such as those outlined above suggest that response to a given antigen will often be produced by either the B-1 or the conventional B cell population, but not both. However, Hayakawa *et al.* (1984) showed many years ago that presenting Dinitrophenol (DNP) on *Brucella abortus* elicited antibody responses from both B-1 and conventional B cells. In a more extensive series of experiments, Wells *et al.* have recently shown that the immune response to $\alpha(1\rightarrow6)$ dextran, unlike the vast majority of the antibody responses studied thus far, is elicited by both B-1 and conventional B cells (S.M. Wells, D. Wang, E.A. Kabat and A.M. Stall, submitted). These latter data demonstrate conclusively that the B-1 and conventional B cell populations can respond to the same antigen and that, following challenge with a defined antigen, both are capable of eliciting an antibody response that is similar with respect to kinetics and isotype profile.



Structural studies that characterize the repertoires of B-1 and conventional B cell populations at various locations in adult and neonatal animals are in their early stages. Differences in $V_H$ gene usage have been reported; however, although the overall picture from these studies is most likely correct, the methodology used leaves some room for question concerning the conclusions reached. Current technology, which allows definitive sequencing of Ig heavy and light chains from individual B cells, will provide a clearer view of these repertoire difference. For example, Ig heavy chain sequence data from individually sorted B cells from the adult peritoneal cavity indicate that the fraction of cells that produce antibodies lacking N-region additions is significantly higher in the B-1a subset that in the conventional B cell subset. The B-1b subset is more similar to conventional B cells. The differences found are consistent with the idea that B-1 cells present in adults tend to have been generated early in development, when pro-B cells do not express terminal deoxynucleotidyl transferase (TdT) and thus are not able to increase antibody diversity by the addition of N-region nucleotides (Kantor *et al.*, 1994; A.B Kantor, C.E. Merrill, L.A. Herzenberg and J.L. Hillson, in preparation).

## ORIGINS OF THE B-CELL LINEAGES

The initial separation of B cells into two lineages was essentially based on differences in how the lineages maintain their number in adult animals. Conventional B cells, being the predominant B cell population in spleen and lymph nodes, are continually replenished by *de novo* differentiation of progenitors in the bone marrow (Hayakawa *et al.*, 1985, 1986). In addition, they can be readily reconstituted in irradiated recipients by transfers of relatively undifferentiated (B220$^-$) adult bone marrow cells but not by transfers of mature conventional B cells in bone marrow or in the periphery (A.B. Kantor, A.M. Stall, S. Adams, K. Watanabe and L.A. Herzenberg, submitted).

B-1a cells, in contrast, are well reconstituted by transfers of fetal and neonatal sources of lymphoid progenitors but not by transfers of adult bone marrow (Hayakawa *et al.*, 1985; Solvason *et al.*, 1991; Kantor *et al.*, 1992). Furthermore, they can be readily reconstituted by transfers of mature Ig$^+$ B-1 cells from the peritoneal cavity (Hayakawa *et al.*, 1986). Thus, unlike conventional B cells, the B-1 population has a substantial capacity for self-replenishment (see above) and therefore is able to maintain its numbers in adults in the absence of continued *de novo* differentiation from early progenitors. The selective lack of B-1a progenitor activity in adult bone marrow indicates that the progenitors for B-1a cells are distinct from progenitors for conventional B cells and hence that these cells belong to separate developmental lineages.

The expression of several 'activation markers', such as CD43, on B-1 cells, the restriction of a particular antibody response to either B-1 or conventional B cell populations, and the apparent skewing of B-1 cell antibody production towards

auto- and anti-bacterial specificities have led others (Rabin *et al.*, 1992; Haughton *et al.*, 1993) to propose that B-1 cells are conventional B cells that have differentiated to the B-1 phenotype in response to activation with a particular type of antigen (i.e. TI type II). This idea has been strongly advocated by Wortis and colleagues, who demonstrated that stimulating conventional B cells *in vitro* with anti-IgM and IL-6 induces expression of CD5 and certain other markers associated with B cell activation (Ying-zi *et al.*, 1991). The authors view this anti-IgM stimulation as a model of TI II responses and interpret the phenotypic shift towards the B-1 phenotype *in vitro* as evidence that the B-1 population seen *in vivo* is generated by stimulating conventional B cells with TI II antigens.

The demonstration that B cell progenitors in bone marrow fail to give rise to more than a few B-1a cells while progenitors from fetal and neonatal animals readily generate these cells would appear to negate this hypothesis. However, proponents of the T type II hypothesis rationalize these developmental differences by postulating that, unlike B-1 cells, bone marrow-derived B cells arise relatively late in life and do not tend to make antibodies that react with TI type II antigens. This selective non-reactivity is ascribed to the N-region insertions that the TdT enzyme introduces into the Ig heavy chains produced by the bone marrow-derived cells (Haughton *et al.*, 1993). These insertions would be rare in B cells generated in young animals since TdT is not expressed in fetal pro-B cells (Li *et al.*, 1993). Thus, following the logic of this hypothesis, conventional B cells generated from progenitors in neonates would be more likely to produce antibodies reactive with TI type II antigens and hence would be more likely to give rise to B-1 cells than conventional B cells generated from (TdT-expressing) progenitors in adult bone marrow.

The antibody responses of B-1 cells and conventional B cells, however, cannot be readily defined in terms of either the form in which the antigen is presented or the characteristics of the epitope to which the response is produced. Nor can the antibodies themselves be defined in terms of particular structural characteristics. For example, as indicated above, some TI and some TD antigens readily stimulate antibody production in B-1 cells; however, others only stimulate antibody production in conventional B cells. Furthermore, as we have recently shown (S.M. Wells, D. Wang, E.A. Kabat and A.M. Stall, submitted) $\alpha(1\rightarrow6)$ dextran, a well-studied TI type II antigen, stimulates antibody secretion by both B-1 and conventional B cells. Finally, also as indicated above, we have recently shown (Kantor *et al.*, 1994; A.B. Kantor, C.E. Merrill, L.A. Herzenberg and J.L Hillson, in preparation) that although B-1a cells tend to have few N-region insertions more frequently than conventional cells, many B-1a cell $V_H$ transcripts have substantial numbers of these insertions. Thus, the weight of the antibody specificity and structure data argues strongly against either of these factors being involved in determining the subset/lineage fate of developing B cells.

While B-1 cells share some phenotypic characteristics with B cells activated following lipopolysaccharide or anti-IgM stimulation, such as lower expression of B220/6B2 and IgD and the absence of CD23, it is too simplistic to characterize them merely as activated B cells. Our recent data indicates that the phenotype of the peritoneal and splenic B-1 cells is distinct from that typically associated with B cells activated with particular protocols. Studies with *in vivo* antigen-activated B-1 cells reveal that the morphology and surface phenotype of B-1 cells is significantly altered following activation (S.M. Wells, A.B. Kantor and A.M. Stall, in preparation). The B-1 cells found in the spleen and peritoneal cavity may have previously interacted with antigen and be in a primed (semi-activated) state ready for further antigenic stimulation.



## The two lineage model

The idea that there are two distinct lymphocyte lineages in the murine immune system, which was initially posited to account for the developmental differences between B-1a and conventional B cells, is supported both by studies of T cell and B cell development. In B cell development, B-1a cells are generated from fetal and neonatal progenitors but not from progenitors in adult bone marrow; in T cell development, the first waves of $\gamma/\delta$ T cells to develop in the neonatal thymus have similarly been shown to derive from progenitors that are present in fetal lymphoid progenitor sources but not in adult bone marrow (Ikuta *et al.*, 1992a,b). Furthermore, both in T cell and B cell development, bone marrow progenitors have been shown to give rise to lymphocytes that are not normally found in large numbers in fetal or neonatal tissues. These developmental patterns suggest that the lymphocytes that develop primarily during fetal life and those that develop primarily in adults may belong to separate lineages derived from independent lymphoid stem cells that have diverged prior to the emergence of progenitors that are committed to differentiate to B or T cells.

Little is known about differences amongst lymphoid stem cells; however, adoptive transfers of various sources of lymphoid progenitors into irradiated recipients have shown a clear locational separation between progenitors for B-1a and conventional B cells. Progenitors that give rise to conventional B cells are present in both the adult bone marrow and the fetal liver. In contrast, B-1a progenitors are rarely present in adult bone marrow, but are readily detectable in the fetal and neonatal liver (Hayakawa *et al.*, 1985; Solvason *et al.*, 1991; Kantor *et al.*, 1992). Furthermore, other early sources of progenitors, such as the fetal omentum and day 9 para-aortic splanchnopleura, give rise to B-1a cells but fail to reconstitute conventional B cells (Solvason *et al.* 1991; Godin *et al.*, 1993); These studies provide strong evidence that the progenitors for B-1a cells are not only distinct from, but also arise earlier in ontogeny than, progenitors for conventional B cells.

The conclusions from these adoptive transfer studies are further supported by the *in vitro* experiments of Hardy and colleagues. These studies show that FACS-sorted fetal pro-B cells cultured on appropriate stromal layers give rise to B-1a cells; however, cultures of similarly sorted adult bone marrow pro-B cells give rise to conventional B cells but not to B-1a cells (Hardy and Hayakawa, 1991). Thus, pro-B

cells, the earliest identifiable cells in the B cell developmental pathway, are already committed to develop into B cells that belong to one or the other lineage.

Until recently, phenotypic distinction between any of the B cell precursors from fetal sources such as fetal and neonatal liver and from adult sources such as adult bone marrow was not possible. However, recent studies by Lam and Stall of MHC class II expression on pre-B cells clearly demonstrate phenotypic differences between fetal and adult B cell development. They show that in contrast to pre-B cells in the adult bone marrow, which express I-A and I-E (Tarlinton, 1994), these class II molecules are absent on the surface of pre-B and newly generated sIgM$^+$ cells in the fetal liver (Lam and Stall, 1994). They further show that the while the 'adult-type' B cell developmental pathway is predominantly found in bone marrow, 'fetal-type' B cell developmental pathway is present in many neonatal lymphoid organs, providing additional evidence of two distinct B cell developmental pathways during ontogeny.

Taken together, the adoptive transfer and *in vitro* studies strongly argue for the existence of two B cell developmental lineages. While these studies cannot rule out the possibility that a conventional B cell could be stimulated to give rise to a cell with a B-1 phenotype, the body of adoptive transfer (Hayakawa *et al.*, 1985; Hardy and Hayakawa, 1992; Kantor *et al.*, 1992) and anti-allotype depletion (Lalor *et al.*, 1989b; K. Watanabe, L.A. Herzenberg and A.B. Kantor, in preparation) experiments indicate that in a normal developing animal B-1 cells are derived predominantly, if not exclusively, from the fetal/neonatal lineage.

## ADDITIONAL B-CELL LINEAGES

While the evidence for separate lineages is strongest for B-1a and conventional B cells other subpopulations of B cells may also represent distinct developmental pathways. As discussed above, stem cell progenitors for B-1a and conventional B cells have not been phenotypically distinguished; however, their temporal and spatial isolation in fetal versus adult lymphoid progenitor sources have allowed the distinction of these lineages. These distinctions are not as clear cut for the B-1b populations. All tissue sources studied thus far have progenitor activity for B-1b cells (Solvason *et al.*, 1991), Godin *et al.*, 1993), although adult bone marrow is deficient when compared to fetal liver (Kantor *et al.*, 1992). Thus, although phenotypic and developmental evidence strongly suggest that B-1b cells constitute a separate lineage derived from independent progenitors present both in fetal lymphoid progenitor sources and in adult bone marrow, the current evidence can also be interpreted as indicating that the progenitors for B-1a cells *and* the progenitors for conventional B cells both give rise to B-1b cells, i.e. that B-1b cells belong to both lineages rather than constituting a lineage of their own.

MZ and follicular B cells have also been proposed as separate B cell lineages



(MacLennan *et al.*, 1982). The functional and anatomical differences between these two B cell subsets argue strongly for this distinction (Kroese *et al.*, 1992; Waldschmidt *et al.*, 1992). However, progenitors from both fetal liver and adult bone marrow equally reconstitute both populations and no source has yet been found to preferentially provide progenitors for one and not the other. Thus the lineage origins of the MZ and follicular B cells are still unclear. Similarly, although Klinman and colleagues have demonstrated functional differences between populations of B cells that give rise to primary and secondary responses (Linton *et al.*, 1989), it is not yet clear whether these represent distinct lineages that can be distinguished within B cell precursor populations.

## THE LAYERED EVOLUTION OF THE IMMUNE SYSTEM

The importance of antibody production to mammalian survival provides a clear rationale for the evolution of the complex processes involved in Ig gene rearrangement, isotype switching and B cell development. Similarly, the evolutionary value of the various functions that T cells perform clearly provides a strong impetus for the evolution of these cells. But while we, as students of the immune system, can readily offer explanations for 'why' it evolved, we have little concept of how.

We have recently pointed out that the existence of distinct B cell and T cell lineages that develop successively during ontogeny may reflect an evolutionary process in which lymphocytes capable of more advanced functions were acquired in layers as mammals evolved from more primitive organisms (Herzenberg and Herzenberg, 1989; Kantor and Herzenberg, 1993). In essence, this model proposes that evolution has created a series of at least two mammalian hematopoietic stem cells that begin functioning sequentially during ontogeny and give rise to lymphocytes (and erythroid and myeloid cells) with progressively more advanced capabilities. Thus, B-1a cells and the first wave of T cells to enter the thymus would be generated from the earliest stem cell, which begins functioning in the mouse during the second trimester of fetal life, whereas follicular and MZ cells would be generated from later stem cells, which also give rise to evolutionarily more mature T cells. The validity of this model is clearly open to question; however, its value in stimulating experiments aimed at distinguishing different types of stem cells and the lymphocyte lineages they engender, as reviewed here and elsewhere (Ikuta *et al.*, 1990, 1992b), is well established.

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