Leonore A. Herzenberg

B-1 cells: the lineage question revisited

Author's address
Leonore A. Herzenberg
Department of Genetics
Stanford University School of Medicine
Stanford CA 94305
USA
Fax: 1 650 725 8564
e-mail: leeherz@darwin.stanford.edu

Summary: The origins and functions of B-1 cells have sparked a good deal of controversy, largely centered on whether these B cells are developmentally distinct from the principal B cell populations (B-2) found in peripheral lymphoid organs. However, the prime criteria for assigning B-1 and B-2 cells to separate developmental lineages are satisfied by studies published some time ago that 1) identify distinct sources of progenitors for B-1 and B-2 cells; 2) show that these progenitors express their inherent commitment developing under the same conditions in co-transfer recipients; and, 3) have distinctive developmental patterns revealed by analysis of cells at various stages along the B-cell development pathway. I review these developmental studies here both to clarify the issue and to set the stage for presentation of evidence from more recent studies, which further define the functional differences between B-1 and B-2 cells and reveal intriguing complexities in the selective and other mechanisms that control the V_H composition of the B-1 antibody repertoire.

Introduction

Since K. Hayakawa and R. Hardy first identified $CD5^+$ (B-1) cells in my laboratory nearly 25 years ago (1–5), the origins and functions of B-1 cells have sparked a good deal of controversy (6–35). These cells, although constituting only a minor fraction of B cells in spleen and lymph nodes of mice, represent the main B-cell population in the peritoneal and pleural cavities. They produce much of the Ig and most of the innate (natural) antibodies in serum and contribute significantly to the IgA-producing plasma cells in the lamina propria of the gut. Nevertheless, they participate in relatively few antigen-stimulated antibody responses, mostly those stimulated by T-independent antigens but some also stimulated by T-dependent antigens (1, 36–39).

Consistent with this primitive functional phenotype, key aspects of B-1-cell development differ from the development of B-2 cells, which predominate in spleen and lymph nodes and produce most adaptive (antigen-stimulated) antibody responses. Most notably, B-1 cells develop *de novo* prior to weaning and persist thereafter as a self-replenishing population, whereas the

Immunological Reviews 2000
Vol. 175: 9–22

Immunological Reviews
ISSN 0105-2896

B-2 cells develop *de novo* and continue to enter the mature B-2 pool throughout life (11, 21–24, 40, 41).

The antibody repertoires expressed by B-1 and B-2 cells were originally thought to differ extensively; however, later studies charting V_H sequences expressed by individually sorted B-1 and B-2 cells demonstrated that, by and large, the repertoires these two B cells express are more similar than different (42, 43). Nevertheless, key and quite dramatic differences remain, leading several groups to propose that the phenotypic and functional distinctions between B-1 and B-2 cells are due to antigen-triggered events that drive maturing B cells into one subset or the other (31, 34, 44–46). In support of this argument, substantial evidence exists to indicate that B-2 cells can assume a B-1 phenotype, including expression of CD5, under some circumstances (7, 8, 47–49). However, data from an extensive series of studies in which we and others monitored entry of bone marrow-derived cells into the B-1 pool in adult animals demonstrate that this phenotype switch rarely occurs under normal circumstances (12–14, 22, 37, 40, 41, 50). Thus, one could question the physiologic relevance of being able to turn B-2 cells into B-1 cells.

The decisive reason for treating B-1 and B-2 cells as separate developmental lineages, however, is not based on whether B-2 cells do or do not regularly change to B-1 cells. We are quite willing to grant that B-2 cells may be a *bona fide* source of the small number of B-1 cells that derive from bone marrow under our observation conditions. The key issue is whether the two kinds of B cells normally derive from distinct progenitors whose lineage commitment is established early in development and is maintained even when the progenitors are forced to develop in the same environment.

This prime criterion for assigning cells to separate lineages is satisfied by studies that 1) identify distinct sources of progenitors for B-1 and B-2 cells; 2) show that these progenitors express their inherent commitment developing under the same conditions in co-transfer recipients; and 3) have distinctive developmental patterns revealed by analysis of cells at various stages along the B-cell development pathway. We review these developmental studies here to clarify the issue and set the stage for more recent studies, which further define the functional differences between B-1 and B-2 cells and reveal intriguing complexities in the selective and other mechanisms that control the composition of the B-1 repertoire.

B-1 and B-2 cells derive from distinct progenitors

As indicated above, distinguishing developmental lineages requires demonstration that the cells in two putative lineages derive from distinct progenitors that maintain their respective commitments when they develop in the same environment. The following evidence collectively demonstrates that progenitors for B-1 and B-2 cells satisfy this key criterion:

1) B-2 progenitors in adult bone marrow maintain their commitment to B-2 development *in situ* and in adoptive recipients.
   a) B-cell depletion chimeras. Treating neonatal mice with antibody to the endogenous IgM allotype to remove all B cells and replacing the depleted B-1 cells by injecting mature allotype-congenic B-1 cells (non-reactive with the treatment antibody) during the 6–8 week treatment period creates stable B-cell chimeras in which all B-2 cells are derived from endogenous progenitors but only a small proportion of B-1 cells (mainly B-1b) are endogenously derived (37, 40, 41, 50).
   Whether examined weeks, months or over a year after treatment terminates, the proportion of B-1 cells derived from endogenous progenitors is always small and shows little evidence of the continued input from the progenitors that continually replenish the B-2 population.
   b) Radiation chimeras. Reconstituting lethally irradiated mice with adult bone marrow and either mature allotype-congenic B-1 cells or sources of early progenitors for allotype-congenic B-1 cells results in stable chimeras in which bone marrow contributes minimally to the B-1 population, even when examined a year after reconstitution is complete (3, 22–25, 28, 51, 52). In chimeras made by reconstituting with adult bone marrow and allotype-congenic fetal liver (day 12, $B22^-$), B-2 cells are derived both from bone marrow and fetal liver, whereas B-1 cells are derived almost exclusively from fetal liver.
2) The failure to derive significant numbers of B-1 cells from adult bone marrow is not due to regulatory mechanisms that obscure B-1 progenitor activity.
   a) Bone marrow does not contain inhibitors that mask the activity of B-1 progenitors, since the B-1 progenitors in fetal liver identically reconstitute B-1 cells in the presence or absence of adult bone marrow (22–24, 53).
   b) Fetal liver does not contain cells required to support B-1 development (e.g. from progenitors in bone marrow), since the small number of B-1 cells derived from bone marrow in adoptive recipients is not increased by co-transfer of fetal liver despite the full development

of a B-1 population from fetal liver in the same recipient (22–24, 53).

3) Progenitors for B-1 cells are selectively lost when neonatal B cells are depleted for 6 weeks (by treatment with anti-IgM)* (37, 40, 41, 51).
   a) B-2 recovery begins as soon as the treatment antibody disappears and goes to completion.
   b) B-1 recovery in the same animals either fails entirely or is significantly disrupted, depending on whether the treatment removed all B-1 cells or only the (allotype-marked) B-1 cells recognized by the treatment antibody.
4) Progenitors for B-1 cells are distinguishable from progenitors for B-2 cells and maintain their commitment in adoptive recipients.
   a) Embryonic splanchnopleura and fetal omentum contain progenitors that reconstitute B-1, but not B-2, cells in adoptive recipients (54–56).
   b) Progenitors that reconstitute B-2 cells are found in fetal liver and at neonatal sites, including bone marrow. Progenitors for B-1 cells are also found in these locations (22).
   c) The B-1 progenitors in neonatal animals are largely gone by the time the animals are weaned (2–3 weeks of age). Progenitors for B-2 cells, in contrast, persist or increase in frequency in bone marrow as animals mature after weaning. Thus, while B-1 cells are poorly reconstituted by adult bone marrow (sometimes not at all), progenitors for B-2 are abundant at this site and readily reconstitute the full adult B-2 population (22).

The relationship between the B-1 and B-2 progenitors in fetal liver is still somewhat cloudy. The fetal omentum is contiguous with the liver capsule. Therefore, as day 13 fetal omentum contains B-1 but not B-2 progenitors (55, 56), it is possible that some or all of the B-1 progenitors detected in fetal liver at the same time (or earlier) are located in the liver capsule or in co-isolated omental tissue. The B-2 progenitors, in contrast, are found in the fetal liver (22, 55, 56) but not in the omentum. Thus, B-2 progenitors may arise solely from hematopoetic progenitors that populate the interior of the fetal liver and later populate the bone marrow. Resolution of this issue awaits the development of methods either for sorting fetal B-1 from B-2 progenitors or for isolating the fetal liver capsule from the cells in the liver interior.

* These findings are described more fully in a later section.

The B-1 and B-2 developmental pathways differ

Several studies contrasting the phenotype and progenitor potential of cells at various stages along the bone marrow B-cell development pathway with similar stages along the fetal/neonatal B-cell development pathway reveal inherent differences in these pathways.

1) Pro-B cells isolated from fetal liver give rise to B-1 cells *in vitro* whereas pro-B cells isolated from bone marrow do not (16).
2) Differences in the expression of at least one internal protein have been noted between fetal liver and bone marrow pre-B cells (57).
3) MHC class II is expressed on pre-B cells in adult bone marrow and thus precedes expression of surface Ig expression. In contrast, in neonates, Ig expression precedes the expression of class II expression, which occurs at about the same time that IgD and CD5 are expressed on the Bcells (58, 59).

Thus, the collective evidence from phenotypic, functional and co-transfer studies provides ample justification for assigning B-1 and B-2 cells to distinct developmental lineages.

B lineages: a continuing controversy

The evidence discussed above notwithstanding, there is a strong tendency to view the differences between B-1 and B-2 cells as stemming solely from differences in the specificity of the Ig they express (7, 8, 44, 46–49). The basic premise, couched in several ways, is that B-1 and B-2 cells arise from the same progenitor but express Ig receptors whose antigen-binding specificity dictates whether the newly formed B cells are triggered to differentiate to B-1 or to B-2 cells when the cognate antigen is encountered. Antigens encountered during fetal and neonatal life, in this model, drive development largely toward the B-1 phenotype, whereas antigens encountered later in life drive development toward the B-2 phenotype.

This simple model is attractive in that it provides a basis for explaining both developmental and functional (Ig repertoire) differences between B cells. However, it is not consistent with the co-transfer data discussed above, which show definitively that B-cell progenitors from bone marrow and fetal liver developing in the same animal (and hence exposed to the same antigens) follow the inherent developmental pathways imposed by their origins. Distinctive receptor rearrangement, expression and selection patterns may indeed be central to the developmental programs of the individual B-cell lineages. However,

receptor differences cannot be the root cause of the differences between B-1 and B-2 cells since, in the developmental sense, the progenitor commitments discussed above predate the receptor distinctions. This leads us to question the source of the controversy, and to wonder whether reconsideration of the basic issues might help it disappear entirely.

One lineage versus two

In general, the arguments raised in favor of single-lineage models do not directly address progenitor issues. Instead, they focus largely on repertoire issues and on the effects of stimulation with antigen or antigen surrogates on B-cell phenotype. Similarly, arguments supporting the two-lineage models tend to focus on progenitor studies and developmental pathway differences and treat repertoire issues mainly within the context of B-cell function. Thus, the two "camps" have largely been out of communication. I attempt to bridge this gap here by showing how (some of) the single-lineage arguments are interpreted in the context of the two-lineage model. Hopefully, I will be forgiven if I fail to represent the single-lineage arguments as well as their proponents would prefer*.

Differential terminal deoxytransferase (TdT) expression

In one of its best known forms, proponents of the single-lineage model invoke the relatively late expression of the enzyme terminal deoxytransferase (TdT), which introduces N-region insertions during *Ig* rearrangement, to explain why B-1 development is more prevalent during the fetal/neonatal period than in adults. In essence, it is argued, lower TdT levels early in ontogeny will result in the predominant development of B cells expressing germline genes, which have evolved to provide protection against pathogens and thus tend to recognize antigenic determinant expressed on bacteria. Encounter with these types of antigens then triggers differentiation to B-1 cells and/or facilitates entry into the permanent B-1 repertoire. Thus, the theory posits that B cells that develop during fetal (and neonatal) life, when TdT expression is minimal, will tend to become B-1 cells because their receptors have little or no N-region insertion. In contrast, B cells that develop in adult bone marrow, where TdT is fully expressed, will tend to become/remain B-2 cells because their receptors are not well suited to recognize the kinds of antigens that trigger differentiation to B-1 cells.

The differences in TdT expression on which this argument is based are well documented and not at issue. The antigen-mediated conversion of B-2 into B-1 cells is more problematical, but can be granted for the sake of discussion here. (It is discussed more fully below.) The central issue is whether TdT differences introduce significant repertoire differences between B-1 and B-2 cells, and whether such repertoire differences in any event account for differences between the development of B-1 at fetal/neonatal sites and of B-2 cells in adult bone marrow.

On this point, the evidence is clear. Current data demonstrate that N-region differences between B-1 and B-2 are substantially less dramatic than previously assumed (see below). Furthermore, and more importantly, the co-transfer studies discussed in previous sections definitively demonstrate that progenitors from fetal liver fully reconstitute B-1 cells in the same environment (i.e. in adoptive recipients) in which progenitors in co-transferred bone marrow concomitantly fully reconstitute B-2 cells but give rise to few (and sometimes no) B-1 cells. Neither repertoire differences nor differences in antigenic stimulation can account for the differential origins of B-1 and B-2 cells in this common environment. This finding can only be explained by the existence of unique progenitors with distinctive developmental programs in fetal liver and adult bone marrow.

Repertoire differences between B-1 and B-2 cells

The TdT hypothesis and other aspects of repertoire-based support for single-lineage model were (and in some cases still are) fueled initially by what later turned out to be faulty repertoire data that we and our colleagues nonetheless accepted as valid at the time. In essence, the hybridoma and bulk Ig sequence data that were available indicated that B-1 cells mainly express IgH encoded by the Q52, 7183 and V_H11 gene families while B-2 cells mainly express Ig encoded by J558 V_H family. In addition, these early sequence data indicated that receptors expressed by B-1 cells only rarely have N-region insertions, whereas receptors expressed by B-2 cells nearly always have such insertions (60–63). These findings created the impression that there is very little overlap in the V_H gene expression in the Ig repertoires expressed by B-1 and B-2 cells (46, 61, 64–67).

Recent repertoire studies, however, show that the earlier methods (hybridoma analyses, bulk cDNA, etc.) greatly overestimated the differences between the B-1 and B-2 repertoires. Single-cell RT-PCR studies characterizing V_H and V_L gene expression and N-region insertion in individual FACS-sorted B-1 and

* In addition, I hope I will be forgiven for citations I may have omitted. I have included many, but I am sure there are several others that have escaped me for the moment. The reviews cited here hopefully will provide the additional resources necessary to find all of the relevant material.

B-2 cells (42) now show that although there are clear V_H usage differences between the B-1 and B-2 repertoires, the overall V_H usage is relatively similar. Similarly, although there are more B-1 than B-2 cells expressing sequences with no N-region insertion at either the *DH* or *JH* junction, the difference is not nearly as dramatic as previously supposed. Roughly 5% of B-2 cells and 30 % of B-1 cells fall into this category, while the majority of cells in both lineages express Ig with N-region insertion in at least one junction (42, 43, 68). Nevertheless, current Ig sequence data offers substantially less comfort to proponents of single-lineage models.

Selection

More modern theories focus on the selective expression of certain V_H genes in the B-1 repertoire and the ways that cells expressing these genes are either selected into the B-1 pool or triggered to differentiate into B-1 cells. The differences between the B-1 and B-2 repertoires are certainly consistent with the idea that receptor specificity plays a major role in determining whether cells enter the B-1 or B-2 pool. However, these differences are also consistent with the existence of separate lineages whose individual development programs favor the entry or retention of Bcells that express certain types of receptors.

Hardy and colleagues, in fact, have directly demonstrated that the B-cell development pathway followed by progenitors in fetal and neonatal animals results in the entry/retention of B cells with different types of receptors than the B cells generated by the development pathway in adult bone marrow (52, 69). These studies show that the well-established selection mechanisms responsible for persistence and expansion of pre-B cells in adult bone marrow are reversed in the fetal/neonatal pathway such that production of immunoglobulin μ heavy chain and subsequent assembly with a surrogate light chain to form the pre-B-cell receptor complex inhibits pre-B-cell growth in fetal liver. Thus, while pre-B cells expressing V_H that do not associate efficiently with surrogate light chain tend to be lost in bone marrow, their survival and further maturation is favored in fetal liver.

The Hardy findings introduce a key mechanistic difference that influences the B-cell repertoires generated during the fetal/neonatal period as opposed to the adult period. If we assume (as seems likely) that the same mechanism operates in fetal liver/bone marrow chimeras, then we can expect that the B-1 and B-2 repertoires in the chimeras will differ even though they develop in the same environment. This hypothesis is currently being tested in our laboratory.

In any event, from a logical perspective, neither end-point analyses (i.e. repertoire composition) nor demonstrations that receptors with certain specificities are selected into one or the other lineage can distinguish between the one-lineage and the two-lineage model. All models agree that Ig structure plays a major role in determining entry or retention in the B-1 and B-2 pools, and hence that there must be mechanisms that mediate these entry/retention differences. Single-lineage adherents argue that these mechanisms are all there is. Two-lineage adherents argue that the differences observed by Hardy and colleagues reflect inherently different developmental mechanisms that result in repertoire differences. Until the characterization of the repertoire in the fetal liver/bone marrow chimeras is complete, there is still at least a little room to argue that the observed repertoire differences are environmentally dictated rather than inherent in the progenitor's developmental program. Nevertheless, regardless of how the repertoire differences between B-1 and B-2 cells are generated, the progenitor studies still stand as a straightforward demonstration that these cells belong to different developmental lineages.

Phenotypic conversion of B-2 to B-1

Evidence indicating B-2 cells can be triggered to shift to a B-1 phenotype similarly does not detract from the basic conclusion that B-1 and B-2 cells normally arise from independent progenitors and thus belong to separate lineages. Wortis and colleagues, for example, have identified *in vitro* conditions that stimulate B-2 cells to express B-1 phenotypic characteristics (7). In addition, Rajewsky and colleagues have reported that when the Cre-lox system is used *in vivo* to switch the transgenic Ig receptor expressed on B-2 cells to a typical B-1 receptor, there is a rapid increase in splenic B cells expressing the B-1 phenotype that are apparently derived directly from the B-2 cells that express the "switched-in" receptor. These findings are roundly cited as evidence against the two-lineage model; however, they basically provide information about what B-2 cells can do, not what they normally do (8, 49).

In essence, "conversion" findings such as these may be highly informative with respect to the developmental potential inherent in B-2 cells. However, they do not seriously challenge the two-lineage model, which (as indicated above) is solidly based on the identification of distinct progenitors for each lineage and the demonstration that there is minimal conversion between the lineages under normal circumstances. At best, since there are usually a few B-1 cells that derive from bone marrow in adoptive recipients, the demonstration that phenotypic conversion from B-2 to B-1 is possible may explain the

origins of a very small proportion of B-1 cells in the normal animal.

Transgenic models

Data from studies with a broad series of transgenic and gene-targeted mice in which B-1 development is selectively disrupted have also been advanced as evidence favoring the single-lineage model. The reasons that these defects interfere with B-1 development or alter B-1 frequencies are clearly interesting and well worthy of study. However, once again, these findings provide scenarios that are reasonable, but offer no convincing evidence to indicate that the mechanisms putatively revealed with genetically altered mice actually operate at significant levels in adult animals.

In sum, although diverse mechanisms may result in the development of B-1 cells in some cases, these forces cannot be invoked to explain the separate origins of B-1 and B-2 cells demonstrated by the bone marrow/fetal liver co-transfer studies. Even if differences in the expressed Ig receptors were shown to determine whether cells expressed B-1 or B-2-cell characteristics, the data would have to be interpreted to mean that progenitors for B-1 and B-2 are differentially programed with respect to the range of Ig receptors their progeny are permitted to express. Thus, unless the co-transfer and related studies demonstrating the independence of the progenitors for B-1 and B-2 cells can be shown to be fallacious, the controversy surrounding the lineage origins of B-1 and B-2 cells reduces to a failure either to acknowledge or to appropriately integrate this definitive study.

Feedback inhibition of B-1 development

The decided lack of progenitors for B-1 cells in adult bone marrow (particularly for B-1a cells), coupled with the ability of the B-1 population to replenish itself through successively transferred hosts, provided the first indication that the B-1 population develops *de novo* from progenitors in the neonate and persists thereafter solely by self-replenishment. The proof for this proposition (discussed in detail below) stems from studies in which we differentially depleted B cells from neonatal animals and showed that B-1 cells that develop and persist during the treatment period persist throughout life, whereas the depleted B-1 cells never recover if the depletion conditions are maintained for 6–8 weeks. Findings from these and related studies also reveal the existence of a feedback mechanism through which mature B-1 cells prevent *de novo* B-1 maturation (37, 40, 41, 50).

To differentially deplete Bcells in these studies, we treated neonates with antibodies to IgM heavy chain allotypes (allelic forms of IgM). Treating allotype homozygotes with these antibodies empties all B-cell compartments. Treating allotype heterozygotes, in contrast, depletes only those B cells that express the IgM allotype recognized by the treatment antibody. The remaining B cells, which express the other parental allotype, develop normally and essentially fill the B-cell compartments in the treated animal.

When the treatment is terminated and the treatment antibody disappears, B-2-cell development recovers rapidly in the bone marrow, and immature B-2 cells appear in the periphery within a few days. The mature compartments then fill rapidly in the antibody-treated homozygotes (where all B cells were depleted) and substantially more slowly, at a rate consistent with the turnover rate for splenic B cells, in the allotype heterozygotes (where the spleen has already been filled by B cells expressing IgM that did not react with the treatment antibody). In both types of animals, however, the frequencies of the initially depleted B-2 cells ultimately reach normal levels in all compartments (70, 71).

The rules governing the recovery of the B-1 population are more complex. If the antibody treatment is terminated prior to weaning (2–3 weeks of age), the B-1 population will develop normally. If the treatment is maintained for 6 weeks, the effect on the B-1 population will depend on whether the treatment depletes all of the B-1 cells. In animals that have no B-1 cells when the treatment is terminated, the B-1 population recovers its normal size but not its normal composition in that the proportion of B-1 cells (B-1b) that do not express CD5 is increased. Even if the antibody treatment is maintained for several months, the B-1 population will still recover. However, the longer the antibody treatment is maintained, the higher the proportion of B-1b cells that will be found in the recovered B-1 population.

In animals that have B-1 cells at the time the antibody treatment terminates, the B-1 recovery pattern is governed by a B-cell developmental feedback mechanism similar to that described in the chicken (72, 73). In essence, the presence of mature, self-replenishing B-1 cells, endogenously derived or introduced, blocks almost all subsequent B-1 development. This developmental block is strongest in Ig allotype heterozygotes, where treatment with antibodies to only one parental allotype leaves the endogenous B-1 population expressing the other parental allotype fully intact. However, it is also readily demonstrable in allotype homozygotes by depleting these mice with anti-allotype antibodies that deplete all B cells and introducing mature allotype congenic B-1 cells during the neonatal

treatment period. In both cases, the depleted B-1 cells never recover and the B-1 compartment is populated throughout life by the self-replenishing progeny of endogenous (or transferred) B-1 cells that were not affected by the neonatal anti-allotype treatment.

B-1 cells recover readily when antibody treatment is terminated before weaning. They also recover readily whenever antibody-treated animals have no B-1 cells at the time the treatment is terminated. We therefore conclude from these feedback regulation studies that B-1 cells normally differentiate *de novo* from progenitors during neonatal life to create a functional B-1 population that blocks subsequent *de novo* B-1 differentiation once the neonates are weaned.

In addition to revealing an evolutionarily conserved feedback mechanism controlling B-1 development, these findings open the way to the development of an effective model for evaluating the contribution of B-1 and B-2 cells to innate and adaptive immunity. By depleting B cells from neonatal allotype homozygotes and introducing mature B-1 cells that express a different allotype, long-term B-cell chimeras can be created in which the B-1 and B-2 cells and their antibody products are marked with different allotypes. Our recent studies defining the lineage origins of the B cells that produce innate versus adaptive antibodies reactive with influenza virus (50) use this approach to good advantage (see below).

A third B-cell lineage in the mouse?

The B-1 population contains two types of similar, but nonetheless phenotypically distinct, B-cell populations: B-1a cells, which express low levels of CD5, and B-1b cells, which do not detectably express CD5. FACS-sorted B-1a cells predominantly reconstitute B-1a cells in adoptive recipients and give rise to only as many cells from the other population as can be accounted for by contamination of the sorted cells (27, 53, 74). Similarly, FACS-sorted B-1b predominantly reconstitute B-1b (18, 20). Thus, the characteristics of the two types of cells are heritable and persist through major expansions in adoptive recipients.

The anatomical location, phenotypic characteristics, self-replenishment capability and sensitivity to feedback inhibition of B-1b cells clearly place them within the B-1 lineage. However, bone marrow reconstitution studies suggest the B-1a and B-1 b actually derive from different progenitors (21–23, 53). In essence, while B-1a cells are very poorly reconstituted from progenitors in adult bone marrow, B-1b cells are sometimes reconstituted to half their normal frequency, particularly when bone marrow is transferred alone. Since B-1b typically represents 5–10% of the overall B-1 population, this B-1b reconstitution is not very dramatic numerically. Nevertheless, its substantially greater efficiency in comparison with B-1a suggests that it may derive from progenitors with a significantly different developmental program and thus represent an independent lineage.

Characteristic developmental differences also distinguish the two types of cells. B-1b cells normally appear later than B-1a during development and represent only a small proportion of the B-1 population. However, when *de novo* B-1 development is blocked for the first few weeks or months of life but later enabled, B-1b emerge as the predominant population, e.g. when neonates are depleted of all B-1 cells for 6–8 weeks or longer and the native B-1 population is then allowed to recover (40, 41). In addition, B-1b predominate in the quite small B-1 population that often appears when irradiated animals are reconstituted with bone marrow, particularly in the absence of co-transferred B-1 cells. B-1b cells are sometimes reconstituted to half their normal frequency under these conditions while B-1a cells are usually reconstituted to only 1–5% of normal (21–23, 53). Thus, while neither B-1a or B-1b is well reconstituted by bone marrow, B-1b reconstitution is substantially more successful from this source.

As the name B-1b indicates, we were initially somewhat schizophrenic about treating B-1b cells as distinct from the cells in the B-1a lineage, since most of the properties of B-1a and B-1b are similar. Nevertheless, as data presented above indicate, current evidence is most consistent with B-1a and B-1b cells being derived from distinct progenitors committed to significantly different developmental programs. This conclusion is consistent with evidence demonstrating that B-1a and B-1b populations isolated from the same site (PerC) in the same mouse express substantially different Ig repertoires (21, 23). Thus, the preponderance of evidence favors assignment of B-1a and B-1b cells to separate lineages. Nevertheless, to simplify discussion, we tend to treat B-1a and B-1b as components of a single overall B-1 lineage unless distinction between these two kinds of B-1 cells is necessary in a particular context.

A modern view of murine Ig repertoires

In the tradition of good scientific debates, the B-lineage controversy raised intrinsically interesting questions about differences in the Ig repertoires expressed by B-1 and B-2 cells. However, when we turned our attention towards these questions, we soon realized that the methods that had been used to gather what repertoire information existed were problematical. In essence, these methods were (and still are) useful for identify-

ing gene families and associating the expression of these families with particular antibody responses. However, they predictably introduce systematic bias when used to approximate the frequencies at which individual V_H genes are expressed within a given subset/lineage or within a specific antibody response.

Each method has its own weaknesses: hybridoma-based analyses weight repertoires in favor of Ig sequences expressed by cells at an activation state appropriate for hybridoma formation; bulk PCR methods weight the repertoire in favor of sequences from cells with more abundant or more PCR-effective message; in situ hybridization weights in favor of cells with more abundant and/or more avidly hybridizing message; etc. In addition, none of the methods was suitable for V_H frequency analyses on B-cell subsets that might be quite rare and identifiable only with multiparameter FACS methods.

To create an unbiased view of the B-1 and B-2 repertoires and subsets thereof, we therefore developed single-cell RT-PCR methods that would allow efficient identification of the Ig sequences expressed by individual FACS-sorted B cells (45, 75). Results with this methodology (43, 75) confirm the predicted bias in the earlier repertoire data by showing that the overall peritoneal B-1 and B-2 repertoires in adult mice (5–6 months of age) are considerably more similar than previously believed. For example, we and others had predicted that the Q52 and 7183 families would be highly overexpressed in B-1 cells. However, the expression of these families among B-1 cells is only minimally higher than among B-2 cells. N-region insertion is also similar in B-1 and B-2 cells. Although the number and length of the insertions at both the D_H and J_H joints is somewhat higher in B-2 than in B-1, the disparity once again is not as great as expected.

On the other hand, B-1 cells are well known to produce abundant amounts of certain antibodies that are rarely produced by B-2 cells. Antibodies to phosphatidylcholine (PtC) and to certain dextrans are essentially made only by B-1 cells. Similarly, B-1 cells produce antidextran antibodies and the T15-idiotype antibodies that recognize phosphorylcholine (PC) and predominate in both the T-independent and T-dependent responses to PC. Thus, although the overall B-1 and B-2 repertoires are quite similar, the presence of unique populations of antigen-binding and antibody-producing cells qualitatively distinguish the B-1 repertoire from the repertoire expressed in B-2 cells.

V_H expression in anti-PtC antibodies

Cells producing IgM anti-PtC antibodies were originally recognized because they lyse bromelain-treated mouse erythrocytes in a complement-dependent plaque-forming cell (pfc) assay. Haughton and colleagues, however, showed that these cells bind PtC coupled to fluorescein-containing liposomes and developed a FACS assay (35, 76–78) that has enabled a broad range of studies that characterize the distribution, V_H representation and genetics of PtC-binding cells, all of which are B-1 cells (mainly B-1a).

PtC-binding cells represent a relatively large proportion (up to 15%) of peritoneal B-1 cells in all normal mouse strains. They are also found at low frequencies in the spleen, where they are known to differentiate to antibody-producing cells following lipopolysaccharide stimulation. However, despite their ready response to this non-specific stimulation, they neither increase in frequency nor are induced to secrete antibody by exposure to their cognate antigen.

Single-cell RT-PCR analysis of the V_H genes expressed by FACS-sorted PtC-binding cells reveal a unique, genetically controlled V_H expression pattern (79–81). In a given mouse strain, one of three V_H gene families (V_H11, V_H12 and V_HQ52) tends to dominate the repertoire, e.g. V_H11 predominates in PtC-binding cells isolated from C57Bl/6-related mice while V_H12 predominates in C57BL/10-related strains. V_H12 also predominates in adult CB.17, an IgH allotype-congenic strain on the BALB/c background, while V_HQ52 predominates among PtC-binding cells in the BALB/c strain itself.

V_H11 and V_H12 are commonly expressed in VDJ rearrangements that are found repeatedly at relatively high frequencies among PtC-binding cells isolated from separate animals. These "recurrent" rearrangements are also detectable in the same animal, distinguishable in this case because they are embedded in unique *IgH/IgL* combinations that clearly identify them as unique development events. Thus, they must reflect modest expansion of a large number of independently arising B-1 cells rather than extensive expansion of a few highly selected B-1 clones (79, 80).

Genetic regulation of V_H12 expression in PtC-binding cells

The difference in predominant V_H expression between the PtC-binding cells in BALB/c (IgH^a) and C.B/17 (IgH^b) introduced a new perspective on findings from earlier studies in which we detected unexpected differences in the allotype representation among bromelain pfc in the Igh^a/ IgH^b (BALB/c × C.B/17) allotype heterozygotes over 4 months of age. Normally, the two parental allotypes in an allotype heterozygote are expressed equally in antibody responses and in the B-cell population as a whole. However, we surprisingly found that IgH^b anti-PtC pfc outnumber Igh^a pfc approximately 2:1 in (BALB/c × C.B/17)

F1 mice (K. J. Seidl, J. Wilshire, L. A. Herzenberg, unpublished). Thus, our later demonstration that V_HQ52 predominates in BALB/C and V_H12 predominates in C.B/17 mice suggested that the frequency difference between pfc encoded on the two parental chromosomes in the (BALB/c × C.B/17) F1 might be related to the difference in V_H gene expression in PtC-binding cells in the two parental strains.

Our recent studies, which include backcross analyses and genetic mapping studies with a series of BALB/c-derived strains carrying IgV_H cross-overs, confirm this hypothesis. They show further that the increased frequency of IgH^b anti-PtC in the F1 animals is largely due to the increased frequency of V_H12 anti-PtC encoded on the IgH^b (C.B/17-derived) chromosome (J. Wilshire, L. A. Herzenberg, unpublished). This over-representation of IgH^b V_H12, in turn, traces to a single amino acid difference between the V_H12 proteins encoded by alleles carried by the parental strains (44). This allelic difference, a substitution of threonine for alanine at position 21 in framework 1, surprisingly results in the progressive, antigen-dependent increase in the frequency of PtC-binding cells expressing the V_H12 allele encoded on the C.B/17-derived chromosome (J. Wilshire, L. A. Herzenberg, in preparation).

Results from these studies show that the representation of V_H12^{ala} (the BALB/c-derived allele) is roughly equivalent to V_H12^{thr} in neonatal animals. However, while V_H12^{thr} climbs continuously, V_H12^{ala} frequencies and the frequencies of all other PtC-binding cells remain constant throughout life, both with respect to each other and to the overall frequency of peritoneal B-1 cells. Furthermore, the frequency of cells expressing V_H12^{thr} in antibodies that do not bind PtC also remains constant. Only V_H12^{thr} anti-PtC increases as animals age!

These findings, which imply that the V_H12^{thr} anti-PtC increase is due to continued stimulation with an internal antigen for which PtC is a surrogate (or with internal PtC itself), are supported by preliminary evidence indicating that the apparent affinity/avidity of V_H12^{thr} anti-PtC (measured by the ability to bind graded numbers of PtC liposomes) is substantially higher than the apparent affinity/avidity of V_H12^{ala} measured in the same FACS assay. Thus, it is likely that V_H12^{thr} anti-PtC is unique in that it has a high enough affinity/avidity to sense the presence of the stimulating antigen at levels that confer a clear selective advantage (83).

The recognition that $V_H12^{(thr)}$ PtC-binding cells are particularly apt to undergo selective expansion in a sense simply confirms the findings from the well-known series of studies by Haughton, Clarke, Arnold and their colleagues, who have shown clearly that V_H12 is selectively expanded in C57BL/10-related mice (31–34, 46). Since these mice carry the same V_H12^{thr} allele found in C.B/17, there is little surprise that we also find that V_H12^{thr} PtC-binding cells are selectively expanded. However, while the previous studies have largely treated the V_H12 expansion as a model for the mechanisms that determine which cells will enter and thrive in the B-1 repertoire, our studies examining overall V_H expression in PtC-binding cells in (BALB/c × C.B/17)F1 mice bring the unique nature of V_H12^{thr} PtC-binding cells into focus and thus sever the utility of the V_H12 system as a broad model for B-1-cell development.

This conclusion has sharp implications for the lineage issues discussed earlier, since the evidence demonstrating $V_H12^{(thr)}$ selection is one of the cornerstones of the single-lineage model. As we have indicated, data from the progenitor studies argue cogently that B-1 and B-2 cells belong to distinct lineages. However, as we have also indicated, current evidence does not rule out phenotype shifts or other mechanisms that introduce small numbers of B-2 derived cells into the B-1 population. It is entirely possible, within the confines of the current data, that the heightened sensitivity of V_H12^{thr} PtC-binding cells to selection enables small numbers of B-2 cells expressing these receptors to enter the B-1 population via an antigen-triggered phenotype switch or related mechanisms. We have now initiated studies aimed at determining whether this is the case.

As a practical matter, since the frequencies of all of the V_H genes other than V_H12^{thr} that are expressed in the anti-PtC repertoire in BALB/c-related strains are fixed during the first 3 weeks of life and remain constant thereafter, we suggest that these genes constitute a better model for understanding how the B-1 anti-PtC repertoire as a whole is generated and maintained. Collectively, PtC-binding cells expressing these genes represent up to 15% of the B-1 repertoire and, consistent with the two-lineage model, show minimal or no input from bone marrow-derived sources. Therefore, understanding the processes that lead to their rearrangement and acceptance into the B-1 repertoire is likely to provide a reasonable view of the overall mechanisms that regulate B-1 development.

This is not to say that V_H12^{thr} is the only B-1 receptor whose frequencies are affected by selective forces that operate during adult life. Indeed, Hayakawa has shown that B-1 cells producing natural (innate) antibodies reactive with the Thy-1 antigen enter the repertoire only in animals that express Thy-1 (82) while, on the negative side, at least two groups have shown that introduction of cognate antigen can delete B-1 cells (84, 85). Thus, depending on one's perspective, responsiveness to antigenic stimulation represents the rule for B-1 cells while the lack of responsiveness represents the exception, or vice versa. Put another way, the role(s) that antigenic stimulation plays in the life of a B-1 cell appears to depend critically on

the nature of the receptor, the stimulating antigen and the conditions of stimulation.

T-dependent versus T-independent antibody responses

Although (so far as is known) the principal function of all B cells and their progeny is to produce and secrete antibodies, there are significant differences among mature B cells with respect to phenotype, persistence, anatomical location and, as we have emphasized here, lineage origins. There are also significant differences in the kinds of antibodies B cells produce and the kinds of stimulations that induce production of particular antibodies. Aligning these functional distinctions within the overall B-cell classification scheme has not proven easy. Generalizations abound, but exceptions are prevalent and must be given precedence if an accurate alignment is to be achieved. Therefore, at the moment, we are faced with a basically outdated set of rules and a series of findings that probably do not as yet provide enough information for a more comprehensive analysis.

B-1 and B-2 antibody responses, for example, are thought to separate along functional lines according to whether T-cell help is required for the response. B-1 cells, in this construction, are usually classified as producing T-independent responses, while B-2 cells are classified as producing T-dependent responses. However, there is clear evidence demonstrating that B-1 cells produce both T-dependent and T-independent responses, even to the same determinant presented on different carriers (37, 38, 86).

In the best studied example, B-1 cells produce a T-dependent response to PC coupled to keyhole limpet hemocyanin (KLH) and a T-independent response to PC presented in its native form on heat-killed vaccine of rough *Pneumococcus pneumoniae* R36A (Pn) (38). The predominant antibody in these responses is encoded by *VH-1* and *V kappa* 22 genes and uniformly expresses the T15 idiotype. B-1 cells expressing the T15 idiotype increase in frequency in spleen after PC-KLH immunization and differentiate shortly thereafter to plasma cells that produce typical T15-marked anti-PC antibodies (B. Devlin, A. M. Stall, L. A. Herzenberg, in preparation). Thus, there is little question that B-1 cells can and do produce certainT-dependent responses.

On the other hand, there is also little question that most T-dependent responses are produced by B-2 cells. In fact, B-2 rather than B-1 cells produce the anti-KLH antibodies in animals immunized with PC-KLH. It is not at all clear how this split in the cells responsible for antibody production to separate determinants on the same antigenic molecule occurs. However, that it does occur indicates that there are as yet unknown mechanisms that determine whether B-1 or B-2 cells (or both) will respond to particular antigens or components thereof.

Overall, B-1 cells appear to participate in moreT-independent than T-dependent responses. The demonstration that B-1 responses to PC can be stimulated by PC presented either in a T-dependent or a T-independent format, however, suggests that the tendency toward T-independent responses could reflect the form in which the antigens to which B-1 cells respond (e.g. α-1,3 and α-1,6 dextrans and SIII pneumococcal polysaccharide) are usually presented. It is interesting to speculate whether presentation of the relevant antigenic determinants in a T-dependent format would result in T-dependent responses. If so, then the issue would resolve to a tendency for the B-1 repertoire to contain receptors of the appropriate specificity.

Isotype switching

Although B-1 cells are commonly reputed to produce only IgM antibodies, a large proportion of the IgA-producing cells in the gut have been shown to be derived from B-1 cells (87, 88) and, similarly, half of the serum IgA is B-1 derived (50). Furthermore, a significant amount of the spontaneously produced (innate) IgG in serum is produced by isotype-switched progeny of B-1 cells. In general, within the B-1 contribution to serum, IgG2a>IgG2b>>IgG1 and virtually no IgE is detectable (40, 41). Tarlinton et al. have established that B-1a cells can switch to IgG1 production (22, 89). Furthermore, Vink et al. have shown that when B-1b cells are expanded/activated by treating animals with interleukin-9, serum IgG1 and IgE increase markedly whereas IgG2a and IgG2b remain relatively constant (90). Thus, B-1a and B-1b together produce the full range of isotypes although each lineage appears to have a specialized role with respect to the isotypes it will produce.

Innate antibody production

Although B-1 cells participate in a wide variety of antigen-stimulated responses, ranging from the anti-PC-response discussed above to the specific complement-binding antibodies involved in delayed-type hypersensitivity (91), they are also the source for much (perhaps all) of the spontaneously produced innate (natural) antibody (91), which appears in serum without any apparent antigenic stimulus and whose production often remains unaltered despite introduction of a strong antigenic stimulus.

For example, using B-1/B-2 allotype chimeras to track the sources of serum antibodies, we have recently shown that B-1

cells produce virtually all of the innate antibodies to influenza virus (flu) (50). These antibodies, which cannot have arisen from a previous encounter with the virus since mice are not natural hosts for this pathogen, are present in serum prior to infection with the virus and show no change in levels following intranasal flu infection. The infection clearly stimulates a sharp increase in antiflu antibody production; however, this adaptive response is produced entirely by B-2 cells (50). Thus, the B-2 antiflu response peaks and decays with typical primary response kinetics while the levels of innate antiflu antibodies remain constant.

Importantly, despite this apparent lack of participation in the antiflu response, the steady-state production of natural IgM antibodies by the B-1 cells is crucial for maximal immune protection from infection with this virus. Thus, mortality due to flu infection is increased in chimeric animals in which the B-2 population is derived from wild-type mice and the B-1 population is derived from gene-targeted (knockout) mice ($sIgM^{-/-}$) that can produce surface, but not secreted, IgM. Constructing the chimeras in the opposite direction to enable innate antibody production by B-1 cells and prevent the B-2 adaptive response to the infection results in a similar increase in mortality. In fact, mortality rates in both types of chimeric mice are similar to the mortality rates in intact gene-targeted mice, in which none of the B cells secrete IgM, and are greatly increased compared to mice reconstituted with wild-type B-1 and B-2 cells. Therefore, while the adaptive virus-specific IgM response produced by B-2 cells is predictably central to protection against flu infection, the production of innate IgM antiflu antibodies by B-1 cells also emerges as a crucial non-redundant component of the humoral immune system in that it cannot be replaced by the induction of virus-specific IgM response produced by B-2 cells (N. Baumgarth, O. Herman, G. Jager, L. Brown, M. Boes, L. Herzenberg, J. Chen, submitted).

Interestingly, secreted IgM antibodies have a strong effect on the magnitude of the virus-specific IgG response and thus seem to also affect neutralization of the virus indirectly by promoting induction of the specific IgG response (83) (N. Baumgarth, O. Herman, G. Jager, L. Brown, M. Boes, L. Herzenberg, J. Chen, submitted). This was revealed by the reduced serum levels of virus-specific IgG1 and IgG2a antibodies in $sIgM^{-/-}$ mice compared to their wild-type controls. Consistent with the survival data, both B-1 and B-2-cell-derived IgM are required for establishing a maximal IgG response (92–94), e.g. reduced IgG serum levels are seen in irradiation chimeras that lack secretion by B-1, B-2 or by both cell types. Local production of B-1-cell-derived natural IgM may be of importance in particular for establishing antibody–antigen complexes that, displayed on follicular dendritic cells, promote induction of the B-cell response. However, Baumgarth has proposed that circulating natural IgM may directly affect B-cell activation (95).

Layered evolution of the immune system

Some time ago, we pointed out that the crucial role of the immune system in protecting organisms against invasion by pathogens makes it very difficult for evolution to "experiment" with new avenues of immune responsiveness unless such experimentation is undertaken without interfering with ongoing protection offered by the existing system. To accomplish this goal, we suggested that the evolution of the immune system occurred in layers, with a new layer being put into place before the old layer is superannuated (23, 27, 29). Ultimately, one could expect the new and the older layer to adjust to each other, with some functions remaining in the old layer in a position to back up and co-operate with the newly evolved functions in the latest layer.

Since each layer in such a system would have to be capable of developing and functioning on its own while a new layer is "under construction", it would be difficult (although not impossible) to derive all layers from a single developmental lineage. Thus, just as ontogeny recapitulates phylogeny, one could expect to find that the highly evolved mammalian immune system is composed of functionally distinct layers, embodied in separate developmental lineages, that recapitulate the progressive evolution of phenotype and function. Since the B-1 cells arise earliest in development and display many of the characteristics of primitive B cells, such as those in the chicken (e.g. neonatal development, self-replenishment in adults, feedback regulation of development CD5 expression), we suggested that B-1 cells represent the early evolutionary layer(s) of the immune system whereas B-2 cells represent the more highly evolved later layers (24, 27, 29). The data discussed here remain consistent with this hypothesis.

References

1. Hayakawa K, Hardy RR, Parks DR, Herzenberg, LA. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. J Exp Med;**157**:202–218.
2. Hayakawa K, Hardy RR, Honda M, Herzenberg LA. Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. Proc Natl Acad Sci USA 1984;**81**:2494.
3. Hayakawa K, Hardy RR, Herzenberg LA, Herzenberg LA. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. J Exp Med 1985;**161**:1554–1568.
4. Hardy RR, Hayakawa K, Parks DR, Herzenberg LA. Murine B cell differentiation lineages. J Exp Med 1984;**159**:1169–1188.
5. Hardy RR, Hayakawa K. Development and physiology of Ly-1 B and its human homolog, Leu-1 B. Immunol Rev 1986;**93**:53–79.
6. Wortis HH. Surface markers, heavy chain sequences and B cell lineages. Int Rev Immunol 1992;**8**:235–246.
7. Wortis HH, Teutsch M, Higer M, Zheng J, Parker DC. B-cell activation by crosslinking of surface IgM or ligation of CD40 involves alternative signal pathways and results in different B-cell phenotypes. Proc Natl Acad Sci USA 1995;**92**:3348–3352.
8. Lam KP, Rajewsky K. B cell antigen receptor specificity and surface density together determine B-1 versus B-2 cell development. J Exp Med 1999;**190**:471–477.
9. Haughton G, Arnold LW, Whitmore AC, Clarke SH. B-1 cells are made, not born. Immunol Today 1993;**14**:84–91.
10. Lam K, Stall AM, Kantor AB, Herzenberg LA. Origins of B cell lineages: aspects of the difference between B-1 and conventional B cells in M54 μ transgenic mice. In: Saito H, et al. eds. Molecular basis of immune responses. Proceedings of the First Green Cross International Symposium; 1992 Nov 28–29; Tokyo, Japan. Tokyo: The Naito Foundation for Medical Research through Academic Press/Harcourt Brace Jovanovich Japan, Inc. 1993. p. 39–48.
11. Kroese FG, Ammerlaan WA, Deenen GJ. Location and function of B-cell lineages. Ann N Y Acad Sci 1992;**651**:44–58.
12. Hardy RR. B cell ontogeny and B cell subsets. Curr Opin Immunol 1989;**2**:189–198.
13. Hardy RR. Development of murine B cell subpopulations. Semin Immunol 1990;**2**:197–206.
14. Hardy RR, Carmack CE, Li YS, Hayakawa K. Distinctive developmental origins and specificities of murine CD5$^+$ B cells. Immunol Rev 1994;**137**:91–118.
15. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. Resolution and characterization of pro-B and pre-pro-B-cell stages in normal mouse bone marrow. J Exp Med 1993;**173**:1213–1225.
16. Hardy RR, Hayakawa K. A developmental switch in B lymphopoiesis. Proc Natl Acad Sci USA 1991;**88**:11550–11554.
17. Hardy RR, Hayakawa K. CD5 B cells, a fetal B cell lineage. Adv Immunol 1994;**55**:297–339.
18. Stall AM, Wells SM, Lam KP. B-1 cells: unique origins and functions. Semin Immunol 1996;**8**:45–59.
19. Stall AM, Lalor PA, Herzenberg LA, Herzenberg LA. Ly-1 B cells and autoantibodies. Ann Immunol Inst Pasteur 1986;**137D**: 173–175.
20. Stall AM, Adams S, Herzenberg LA, Kantor AB. Characteristics and development of the murine B-1b (Ly-1 B sister) cell population. Ann N Y Acad Sci 1992;**651**:33–43.
21. Kantor AB. The development and repertoire of B-1 cells (CD5 B cells). Immunol Today 1992;**13**
22. Kantor AB, Herzenberg LA. Origin of murine B-cell lineages. Annu Rev Immunol 1993;**11**:501–538.
23. Kantor AB, Stall AM, Adams S, Herzenberg LA. Differential development of progenitor activity for three B-cell lineages. Proc Natl Acad Sci USA 1992;**89**:3320–3324.
24. Kantor AB, Stall AM, Adams S, Watanabe K, Herzenberg LA. *De novo* development and self-replenishment of B cells. Int Immunol 1995;**7**:55–68.
25. Hayakawa K, Hardy RR, Stall AM, Herzenberg LA. Immunoglobulin-bearing B cells reconstitute and maintain the murine Ly-1 B cell lineage. Eur J Immunol 1986;**16**:1313–1316.
26. Herzenberg LA, Kantor AB. B-cell lineages exist in the mouse. Immunol Today 1993;**14**:79–83.
27. Herzenberg LA, Kantor AB, Herzenberg LA. Layered evolution in the immune system. A model for the ontogeny and development of multiple lymphocyte lineages. Ann N Y Acad Sci 1992;**65**:1–9.
28. Herzenberg LA, Haughton G, Rajewsky K, eds. CD5 B cells in development and disease. Proceedings of a conference. June 3–6, 1991, Palm Beach Gardens, Florida. Ann N Y Acad Sci 1992;**65**:1–601.
29. Herzenberg LA, Herzenberg LA. Toward a layered immune system. Cell 1989;**59**:953–954.
30. Herzenberg LA, Stall AM, Lalor PA, Sidman C, Moore WA, Parks DR. The Ly-1 B cell lineage. Immunol Rev 1986;**93**:81–102.
31. Arnold LW, McCray SK, Tatu C, Clarke SH. Identification of a precursor to phosphatidyl choline-specific B-1 cells suggesting that B-1 cells differentiate from splenic conventional B cells *in vivo*: cyclosporin A blocks differentiation to B-1. J Immunol 2000;**164**:2924–2930.
32. Tatu C, Ye J, Arnold LW, Clarke SH. Selection at multiple checkpoints focuses V_H12 B cell differentiation toward a single B-1 cell specificity. J Exp Med 1999;**190**:903–914.
33. Clarke SH, Arnold LW. B-1 cell development: evidence for an uncommitted immunoglobulin (Ig)M$^+$ B cell precursor in B-1 cell differentiation. J Exp Med 1998;**187**:1325–1334.
34. Arnold LW, Pennell CA, McCray S, Clarke SH. Development of B-1 cells: segregation of phosphatidyl choline-specific B cells to the B-1 population occurs after immunoglobulin gene expression. J Exp Med 1994;**179**:1585–1595.
35. Pennell CA, Mercolino TJ, Grdina TA, Arnold LW, Haughton G, Clarke SH. Biased immunoglobulin variable region gene expression by Ly-1 B cells due to clonal selection. Eur J Immunol 1989;**19**:1289–1295.
36. Hayakawa K, Hardy RR, Herzenberg LA, Steinberg AD, Herzenberg LA. Ly-1 B: a functionally distinct B-cell subpopulation. In: Progress in Immunology V. Tokyo: Academic Press; 1984. p. 661–668.
37. Hamilton AM, Kearney JF. Effects of IgM allotype suppression on serum IgM levels, B-1 and B-2 cells, and antibody responses in allotype heterozygous F1 mice. Dev Immunol 1994;**4**:27–41.
38. Quintans J, Cosenza H. Antibody response to phosphorylcholine *in vitro*. II. Analysis of T-dependent and T-independent responses. Eur J Immunol 1976;**6**:399–405.
39. Kroese FG, Ammerlaan WA, Deenen GJ, Adams S, Herzenberg LA, Kantor AB. A dual origin for IgA plasma cells in the murine small intestine. Adv Exp Med Biol 1995;**371A**: 435–440.
40. Lalor PA, Herzenberg LA, Adams S, Stall AM. Feedback regulation of murine Ly-1 B cell development. Eur J Immunol 1989;**19**:507–513.

cells produce virtually all of the innate antibodies to influenza virus (flu) (50). These antibodies, which cannot have arisen from a previous encounter with the virus since mice are not natural hosts for this pathogen, are present in serum prior to infection with the virus and show no change in levels following intranasal flu infection. The infection clearly stimulates a sharp increase in antiflu antibody production; however, this adaptive response is produced entirely by B-2 cells (50). Thus, the B-2 antiflu response peaks and decays with typical primary response kinetics while the levels of innate antiflu antibodies remain constant.

Importantly, despite this apparent lack of participation in the antiflu response, the steady-state production of natural IgM antibodies by the B-1 cells is crucial for maximal immune protection from infection with this virus. Thus, mortality due to flu infection is increased in chimeric animals in which the B-2 population is derived from wild-type mice and the B-1 population is derived from gene-targeted (knockout) mice ($sIgM^{-/-}$) that can produce surface, but not secreted, IgM. Constructing the chimeras in the opposite direction to enable innate antibody production by B-1 cells and prevent the B-2 adaptive response to the infection results in a similar increase in mortality. In fact, mortality rates in both types of chimeric mice are similar to the mortality rates in intact gene-targeted mice, in which none of the B cells secrete IgM, and are greatly increased compared to mice reconstituted with wild-type B-1 and B-2 cells. Therefore, while the adaptive virus-specific IgM response produced by B-2 cells is predictably central to protection against flu infection, the production of innate IgM antiflu antibodies by B-1 cells also emerges as a crucial non-redundant component of the humoral immune system in that it cannot be replaced by the induction of virus-specific IgM response produced by B-2 cells (N. Baumgarth, O. Herman, G. Jager, L. Brown, M. Boes, L. Herzenberg, J. Chen, submitted).

Interestingly, secreted IgM antibodies have a strong effect on the magnitude of the virus-specific IgG response and thus seem to also affect neutralization of the virus indirectly by promoting induction of the specific IgG response (83) (N. Baumgarth, O. Herman, G. Jager, L. Brown, M. Boes, L. Herzenberg, J. Chen, submitted). This was revealed by the reduced serum levels of virus-specific IgG1 and IgG2a antibodies in $sIgM^{-/-}$ mice compared to their wild-type controls. Consistent with the survival data, both B-1 and B-2-cell-derived IgM are required for establishing a maximal IgG response (92–94), e.g. reduced IgG serum levels are seen in irradiation chimeras that lack secretion by B-1, B-2 or by both cell types. Local production of B-1-cell-derived natural IgM may be of importance in particular for establishing antibody–antigen complexes that, displayed on follicular dendritic cells, promote induction of the B-cell response. However, Baumgarth has proposed that circulating natural IgM may directly affect B-cell activation (95).

Layered evolution of the immune system

Some time ago, we pointed out that the crucial role of the immune system in protecting organisms against invasion by pathogens makes it very difficult for evolution to "experiment" with new avenues of immune responsiveness unless such experimentation is undertaken without interfering with ongoing protection offered by the existing system. To accomplish this goal, we suggested that the evolution of the immune system occurred in layers, with a new layer being put into place before the old layer is superannuated (23, 27, 29). Ultimately, one could expect the new and the older layer to adjust to each other, with some functions remaining in the old layer in a position to back up and co-operate with the newly evolved functions in the latest layer.

Since each layer in such a system would have to be capable of developing and functioning on its own while a new layer is "under construction", it would be difficult (although not impossible) to derive all layers from a single developmental lineage. Thus, just as ontogeny recapitulates phylogeny, one could expect to find that the highly evolved mammalian immune system is composed of functionally distinct layers, embodied in separate developmental lineages, that recapitulate the progressive evolution of phenotype and function. Since the B-1 cells arise earliest in development and display many of the characteristics of primitive B cells, such as those in the chicken (e.g. neonatal development, self-replenishment in adults, feedback regulation of development CD5 expression), we suggested that B-1 cells represent the early evolutionary layer(s) of the immune system whereas B-2 cells represent the more highly evolved later layers (24, 27, 29). The data discussed here remain consistent with this hypothesis.

References

1. Hayakawa K, Hardy RR, Parks DR, Herzenberg, LA. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. J Exp Med;**157**:202–218.
2. Hayakawa K, Hardy RR, Honda M, Herzenberg LA. Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. Proc Natl Acad Sci USA 1984;**81**:2494.
3. Hayakawa K, Hardy RR, Herzenberg LA, Herzenberg LA. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. J Exp Med 1985;**161**:1554–1568.
4. Hardy RR, Hayakawa K, Parks DR, Herzenberg LA. Murine B cell differentiation lineages. J Exp Med 1984;**159**:1169–1188.
5. Hardy RR, Hayakawa K. Development and physiology of Ly-1 B and its human homolog, Leu-1 B. Immunol Rev 1986;**93**:53–79.
6. Wortis HH. Surface markers, heavy chain sequences and B cell lineages. Int Rev Immunol 1992;**8**:235–246.
7. Wortis HH, Teutsch M, Higer M, Zheng J, Parker DC. B-cell activation by crosslinking of surface IgM or ligation of CD40 involves alternative signal pathways and results in different B-cell phenotypes. Proc Natl Acad Sci USA 1995;**92**:3348–3352.
8. Lam KP, Rajewsky K. B cell antigen receptor specificity and surface density together determine B-1 versus B-2 cell development. J Exp Med 1999;**190**:471–477.
9. Haughton G, Arnold LW, Whitmore AC, Clarke SH. B-1 cells are made, not born. Immunol Today 1993;**14**:84–91.
10. Lam K, Stall AM, Kantor AB, Herzenberg LA.
14. Hardy RR, Carmack CE, Li YS, Hayakawa K. Distinctive developmental origins and specificities of murine CD5+ B cells. Immunol Rev 1994;**137**:91–118.
15. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. Resolution and characterization of pro-B and pre-pro-B-cell stages in normal mouse bone marrow. J Exp Med 1993;**173**:1213–1225.
16. Hardy RR, Hayakawa K. A developmental switch in B lymphopoiesis. Proc Natl Acad Sci USA 1991;**88**:11550–11554.
17. Hardy RR, Hayakawa K. CD5 B cells, a fetal B cell lineage. Adv Immunol 1994;**55**:297–339.
18. Stall AM, Wells SM, Lam KP. B-1 cells: unique origins and functions. Semin Immunol 1996;**8**:45–59.
19. Stall AM, Lalor PA, Herzenberg LA, Herzenberg LA. Ly-1 B cells and autoantibodies. Ann Immunol Inst Pasteur 1986;**137D**: 173–175.
20. Stall AM, Adams S, Herzenberg LA, Kantor AB. Characteristics and development of the murine B-1b (Ly-1 B sister) cell population. Ann N Y Acad Sci 1992;**651**:33–43.
21. Kantor AB. The development and repertoire of B-1 cells (CD5 B cells). Immunol Today 1992;**13**
22. Kantor AB, Herzenberg LA. Origin of murine B-cell lineages. Annu Rev Immunol 1993;**11**:501–538.
23. Kantor AB, Stall AM, Adams S, Herzenberg LA. Differential development of progenitor activity for three B-cell lineages. Proc Natl Acad Sci USA 1992;**89**:3320–3324.
29. Herzenberg LA, Herzenberg LA. Toward a layered immune system. Cell 1989;**59**:953–954.
30. Herzenberg LA, Stall AM, Lalor PA, Sidman C, Moore WA, Parks DR. The Ly-1 B cell lineage. Immunol Rev 1986;**93**:81–102.
31. Arnold LW, McCray SK, Tatu C, Clarke SH. Identification of a precursor to phosphatidyl choline-specific B-1 cells suggesting that B-1 cells differentiate from splenic conventional B cells *in vivo*: cyclosporin A blocks differentiation to B-1. J Immunol 2000;**164**:2924–2930.
32. Tatu C, Ye J, Arnold LW, Clarke SH. Selection at multiple checkpoints focuses V_H12 B cell differentiation toward a single B-1 cell specificity. J Exp Med 1999;**190**:903–914.
33. Clarke SH, Arnold LW. B-1 cell development: evidence for an uncommitted immunoglobulin (Ig)M+ B cell precursor in B-1 cell differentiation. J Exp Med 1998;**187**:1325–1334.
34. Arnold LW, Pennell CA, McCray S, Clarke SH. Development of B-1 cells: segregation of phosphatidyl choline-specific B cells to the B-1 population occurs after immunoglobulin gene expression. J Exp Med 1994;**179**:1585–1595.
35. Pennell CA, Mercolino TJ, Grdina TA, Arnold LW, Haughton G, Clarke SH. Biased immunoglobulin variable region gene expression by Ly-1 B cells due to clonal selection. Eur J Immunol 1989;**19**:1289–1295.
36. Hayakawa K, Hardy RR, Herzenberg LA, Steinberg AD, Herzenberg LA. Ly-1 B: a functionally distinct B-cell subpopulation. In: Progress in Immunology V. Tokyo: Academic

41. Lalor PA, Stall AM, Adams S, Herzenberg LA. Permanent alteration of the murine Ly-1 B repertoire due to selective depletion of Ly-1 B cells in neonatal animals. Eur J Immunol 1989;**19**:501–506.
42. Kantor AB, Merrill CE, Herzenberg LA, Hillson JL. An unbiased analysis of V_H–D–J_H sequences from B-1a, B-1b, and conventional B cells. J Immunol 1997;**158**:1175–1186.
43. Kantor AB, Merrill CE, MacKenzie JD, Herzenberg LA, Hillson JL. Development of the antibody repertoire as revealed by single-cell PCR of FACS-sorted B-cell subsets. Ann N Y Acad Sci 1995;**764**:224–227.
44. Haughton G, Arnold LW, Bishop GA, Mercolino TJ. The CH series of murine B cell lymphomas: neoplastic analogues of Ly-1$^+$ normal B cells. Immunol Rev 1986;**93**:35–51.
45. Arnold LW, Haughton G. Autoantibodies to phosphatidylcholine. The murine antibromelain RBC response. Ann N Y Acad Sci 1992;**651**:354–359.
46. Arnold LW, Spencer DH, Clarke SH, Haughton G. Mechanisms that limit the diversity of antibody: three sequentially acting mechanisms that favor the spontaneous production of germline encoded anti-phosphatidyl choline. Int Immunol 1993;**5**:1365–1373.
47. Bandyopadhyay RS, Teutsch MR, Wortis HH. Activation of B-cells by sIgM cross-linking induces accumulation of CD5 mRNA. Curr Top Microbiol Immunol 1995;**194**:219–228.
48. Huang CA, Henry C, Iacomini J, Imanishi-Kari T, Wortis HH. Adult bone marrow contains precursors for CD5$^+$ B cells. Eur J Immunol 1996;**26**:2537–2540.
49. Lam KP, Rajewsky K. Rapid elimination of mature autoreactive B cells demonstrated by Cre-induced change in B cell antigen receptor specificity *in vivo*. Proc Natl Acad Sci USA 1998;**95**:13171–13175.
50. Baumgarth N, Herman OC, Jager GC, Brown L, Herzenberg LA. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. Proc Natl Acad Sci USA 1999;**96**:2250–2255.
51. Hardy RR, Hayakawa K, Parks DR, Herzenberg LA, Herzenberg LA. Murine B cell differentiation lineages. J Exp Med 1984;**159**:1169–1188.
52. Hardy RR, Li YS, Hayakawa K. Distinctive developmental origins and specificities of the CD5$^+$ B-cell subset. Semin Immunol 1996;**8**:37–44.
53. Kantor AB, Stall AM, Adams S, Herzenberg LA. Adoptive transfer of murine B-cell lineages. Ann N Y Acad Sci 1992;**651**:168–169.
54. Godin IE, Garcia-Porrero JA, Coutinho A, Dieterlen-Lievre F, Marcos MA. Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. Nature 1993;**364**:67–70.
55. Solvason N, Lehuen A, Kearney JF. An embryonic source of Ly1 but not conventional B cells. Int Immunol 1991;**3**:543–550.
56. Solvason N, Chen X, Shu F, Kearney JF. The fetal omentum in mice and humans. A site enriched for precursors of CD5 B cells early in development. Ann N Y Acad Sci 1992;**651**:10–20.
57. Oltz EM, et al. A novel regulatory myosin light chain gene distinguishes pre-B cell subsets and is IL-7 inducible. EMBO J 1992;**11**:2759–2767.
58. Lam KP, Stall AM. Major histocompatibility complex class II expression distinguishes two distinct B cell developmental pathways during ontogeny. J Exp Med 1994;**180**:507–516.
59. Hayakawa K, Tarlinton D, Hardy RR. Absence of MHC class II expression distinguishes fetal from adult B lymphopoiesis in mice. J Immunol 1994;**152**:4801–4807.
60. Gu H, Forster I, Rajewsky K. Sequence homologies, N sequence insertion and JH gene utilization in V_H-D-J_H joining: implications for the joining mechanism and the ontogenetic timing of Ly1 B cell and B-CLL progenitor generation. EMBO J 1990;9:2133–2140.
61. Carlsson L, Holmberg D. Genetic basis of the neonatal antibody repertoire: germline V-gene expression and limited N-region diversity. Int Immunol 1990;**2**:639–643.
62. Feeney AJ. Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. J Exp Med 1990;**172**:1377–1390.
63. Meek K, et al. Organization of the murine immunoglobulin V_H complex: placement of two new V_H families (V_H10 and V_H11) and analysis of V_H family clustering and interdigitation. Mol Immunol 1990;**27**:1073–1081.
64. Andersson A, Ekstrand-Hammarstrom B, Eriksson B, Overmo C, Holmberg D. Neonatal treatment with monoclonal natural antibodies restores a normal pattern of *VH* gene utilization in the non-obese diabetic mouse. Int Immunol 1994;**6**:623–630.
65. Carlsson L, Overmo C, Holmberg D. Selection against N-region diversity in immunoglobulin heavy chain variable regions during the development of pre-immune B cell repertoires. Int Immunol 1992;**4**:549–553.
66. Dighiero G, Lymberi P, Holmberg D, Lundquist I, Coutinho A, Avrameas S. High frequency of natural autoantibodies in normal newborn mice. J Immunol 1985;**134**:765–771.
67. Booker JK, Haughton G. Mechanisms that limit the diversity of antibodies. II. Evolutionary conservation of Ig variable region genes which encode naturally occurring autoantibodies. Int Immunol 1994;**6**:1427–1436.
68. Kantor AB, Merrill CE, Hillson JL. Construction of cDNA from single unstimulated mouse B lymphocytes: method and application to the study of expressed antibody repertoires in FACS-sorted murine B cell subsets. In: Herzenberg L, Herzenberg L, Blackwell C, Weir D, eds. The handbook of experimental immunology. Boston: Blackwell Science; 1996. p. 13.1–13.6.
69. Wasserman R, et al. A novel mechanism for B cell repertoire maturation based on response by B cell precursors to pre-B receptor assembly. J Exp Med 1998;**187**:259–264.
70. Cook MC, Basten A, Fazekas de St Groth B. Outer periarteriolar lymphoid sheath arrest and subsequent differentiation of both naive and tolerant immunoglobulin transgenic B cells is determined by B cell receptor occupancy. J Exp Med 1997;**186**:631–643.
71. Cyster JG, Goodnow CC. Tuning antigen receptor signaling by CD22: integrating cues from antigens and the microenvironment. Immunity 1997;**6**:509–517.
72. Pink JR, Ratcliffe MJ, Vainio O. Immunoglobulin-bearing stem cells for clones of B (bursa-derived) lymphocytes. Eur J Immunol 1985;**15**:617–620.
73. Pink JR, Vainio O, Rijnbeek AM. Clones of B lymphocytes in individual follicles of the bursa of Fabricius. Eur J Immunol 1985;**15**:83–87.
74. Herzenberg LA. Murine suppressor T cells: mirage or cloudy reality? Res Immunol 1989;**140**:337–345.
75. Kantor AB, Roederer M. Chapter 49. FACS analysis of leukocytes. In: Herzenberg LA, Weir D, Blackwell C, Herzenberg LA. ed. The handbook of experimental immunology. 5th ed. Boston: Blackwell Science; 1997. p. 49.1–49.13.

76. Mercolino TJ, Arnold LW, Haughton G. Phosphatidyl choline is recognized by a series of Ly-1$^+$ murine B cell lymphomas specific for erythrocyte membranes. J Exp Med 1986;**163**:155–165.
77. Mercolino TJ, Arnold LW, Hawkins LA, Haughton G. Normal mouse peritoneum contains a large population of Ly-1$^+$ (CD5) B cells that recognize phosphatidyl choline. Relationship to cells that secrete hemolytic antibody specific for autologous erythrocytes. J Exp Med 1988;**168**:687–698.
78. Mercolino TJ, et al. Restricted immunoglobulin variable region gene usage by normal Ly-1 (CD5$^+$) B cells that recognize phosphatidyl choline. J Exp Med 1989;**169**:1869–1877.
79. Seidl KJ, et al. Frequent occurrence of identical heavy and light chain Ig rearrangements. Int Immunol 1987;**9**:689–702.
80. Seidl KJ, et al. Recurrent identical rearrangement and repeated expression of identical heavy and light chains in single anti-phosphatidylcholine B cells. Ann N Y Acad Sci 1997;**815**:484-488.
81. Seidl KJ, Wilshire J, MacKenzie JD, Kantor AB, Herzenberg LA, Herzenberg LA. Prominent V_H genes expressed in innate antibodies are associated with distinctive antigen-bindings sites ($J_{(H)}$-CDR3). Proc Natl Acad Sci USA 1999;**96**:2262–2677.
82. Hayakawa K, et al. Positive selection of natural autoreactive B cells. Science 1999;**285**:113–116.
83. Herzenberg LA, Baumgarth N, Wilshire J. B-1 cell origins and V_H repertoire determination. Curr Top Microbiol Immunol (In press).
84. Murakami M, et al. Antigen-induced apoptotic death of Ly-1 B cells responsible for autoimmune disease in transgenic mice. Nature 1992;**357**:77–80.
85. Ohdan H, Yang YG, Shimizu A, Swenson KG, Sykes M. Mixed chimerism induced without lethal conditioning prevents T cell- and anti-Gal α 1,3Gal-mediated graft rejection. J Clin Invest 1999;**104**:281–290.
86. Stohrer R, Kearney JF. Fine idiotype analysis of B cell precursors in the T-dependent and T-independent responses to α 1-3 dextran in BALB/c mice. J Exp Med 1983;**158**:2081–2094.
87. Kroese FG, Butcher EC, Stall AM, Herzenberg LA. A major peritoneal reservoir of precursors for intestinal IgA plasma cells. Immunol Invest 1989;**18**:47–58.
88. Kroese FG, Butcher EC, Stall AM, Lalor PA, Adams S, Herzenberg LA. Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the peritoneal cavity. Int Immunol 1989;**1**:75–84.
89. Tarlinton DM, McLean M, Nossal GJ. B1 and B2 cells differ in their potential to switch immunoglobulin isotype. Eur J Immunol 1995;**25**:3388–3393.
90. Vink A, Warnier G, Brombacher F, Renauld JC. Interleukin 9-induced *in vivo* expansion of the B-1 lymphocyte population. J Exp Med 1999;**189**:1413–1423.
91. Askenase PW, et al. A new paradigm of T cell allergy: requirement for the B-1 cell subset. Int Arch Allergy Immunol 1999;**118**:145–149.
92. Boes M, Esau C, Fischer MB, Schmidt T, Carroll M, Chen J. Enhanced B-1 cell development, but impaired IgG antibody respones in mice deficient in secreted IgM J Immunol 1998;**160**:4776–4787.
93. Boes M, Prodeus AP, Schmidt T, Carroll MC, Chen J. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. J Exp Med 1998;**188**:2381–2386.
94. Ehrenstein MR, O'Keefe TL, Davies SL, Neuberger MS. Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response. Proc Natl Acad Sci USA 1998;**95**:10089–10093.
95. Baumgarth N. A two-phase model of B-cell activation. Immunol Rev (In press).