

Debate: the nature of B-cell subpopulations

It is clear that the B-cell population of normal mice can be divided into subpopulations on the basis of phenotype, anatomical location, ontogeny, antibody repertoire and physiology. But where and how are the subpopulations generated? In this debate, Lee Herzenberg and Aaron Kantor suggest that discrete lineages of B cells, the B-1 (subdivided into B-1a and B-1b) and B-2 (conventional B) populations, are descended from separate precursor cells that are active at different stages of immune system development. In contrast, Geoff Houghton and colleagues propose that the subpopulations develop from a common precursor pool (B-0) into the B-1 and B-3 populations under the influence of antigenic selection (see Box 1). Following comments on the alternative hypotheses by the contributors, the debate is completed by comments from two other leading contributors to the field, Henry Wortis and Richard Hardy.



B-cell lineages exist in the mouse

Leonore A. Herzenberg and Aaron B. Kantor

The idea that the developmental potential of fetal hematopoietic stem cells is identical to the developmental potential of hematopoietic stem cells (HSC) found in adult bone marrow has been around for so long, and has been restated without question in so many textbooks and reviews, that it is generally treated as one of the basic truths of immunology. However, any paradigm based on the identity of two items is only valid until differences are detected between the items. Such paradigms frequently fall when major advances in technology improve the ability to distinguish the items from each other. Thus it is not surprising that the introduction of modern fluorescence analyzers and cell sorters (FACS) and molecular technology, which enabled the recognition of differences in the progeny of fetal and adult stem cells, brought about a major shift in ideas about how the immune system develops.

In essence, recent studies of B-cell development demonstrate that progenitors in adult bone marrow effectively fail to reconstitute B-cell subsets that are readily reconstituted by progenitors in fetal liver and omentum; and, conversely, that progenitors in fetal omentum fail to reconstitute the B-cell subsets that are readily reconstituted by progenitors in adult bone marrow. Similarly, T-cell development studies show that progenitors in adult bone marrow that reconstitute most types of T cells fail to reconstitute one subset

(V γ 3) that is readily reconstituted by progenitors in the fetus. These findings, discussed in more detail below, force replacement of the current paradigm with a multilineage model of the immune system that can accommodate the observed ontological differences in progenitor potential.

Definition of B-cell lineages

A lineage is typically defined as the descent in a line from a common progenitor. Developmental biologists generally adhere to this definition; however, there is often considerable discussion as to what characteristics to use to define a lineage and its progenitor, particularly within the immune system. In the broadest sense, all cells in a given animal can be assigned to a single lineage, since the zygote is the ultimate progenitor; at the other extreme, the progeny of a single, newly-arisen B cell can be treated as a lineage since such B cells are distinguished from each other by unique immunoglobulin (Ig) rearrangements. The most practical definition, however, defines developmental lineages as the set of cells deriving from distinct, relatively undifferentiated (unrearranged) progenitors that have at least a limited capacity for self-renewal and give rise to progeny that are committed to differentiate into cells distinguishable by particular functional or phenotypic characteristics.

On this basis, three murine B-cell lineages are currently recognizable: the 'conventional' B-cell lineage

and the two closely related B-1 lineages, B-1a (formerly Ly-1 B) and B-1b (formerly 'sister') cells^{1,2}. We have just completed a detailed review¹ of the evidence underlying these lineage distinctions and the ways in which this evidence fits with the alternative hypotheses framed to define the origins of B-1 and conventional B cells. We refer the reader to this review for a more satisfactory exposition of the intricacies of the data and hypotheses; however, we welcome the opportunity to summarize our position in this concise 'debate' and to consider new arguments, if they arise.

B-1 versus conventional B cells

The phenotypic characteristics that distinguish the mouse B-cell lineages have been reviewed elsewhere¹⁻⁵. Therefore, we focus here on developmental differences between B-1 and conventional B cells (Table 1). The conventional B-cell lineage contains IgM⁺IgD^{hi} cells that predominate in spleen and lymph node and are replenished throughout life from newly differentiating (unrearranged) progenitors in the bone marrow. These mature B cells can persist for a long time *in situ* and in transfer recipients; however, their numbers do not increase significantly in these recipients^{1,6,7}. Permanent

reconstitution of the overall conventional B-cell population in irradiated recipients clearly requires the transfer of undifferentiated progenitors, such as those found in fetal liver and adult bone marrow and spleen (B220⁺ cells^{8,9}; HSC^{10,11}). The B-1 lineages, in contrast, contain IgM^{hi}IgD⁺ cells that predominate in the peritoneal and pleural cavities. Mature B cells in these lineages survive in adults as a self-replenishing population that can also reconstitute the population when transferred to irradiated or immunocompromised (severe combined immune deficient, SCID) hosts¹⁵. That is, during fetal life B-1 cells begin to develop from progenitors in fetal liver and omentum^{9-10,12}; later, a developmental feedback mechanism becomes active (in 3-6 week old mice^{13,14}) and prevents subsequent *de novo* differentiation of B-1 cells. In effect, the operation of this feedback mechanism results in a virtually total reliance on self-replenishment as the means for maintaining population size in adults.

B-1a versus B-1b

Two key differences distinguish B-1a cells from B-1b cells (Fig. 1). First, although the phenotype of B-1a and B-1b cells is almost identical, B-1a cells express characteristic levels of surface CD5 (Ly-1) molecules whereas B-1b cells do not². Secondly, B-1a cells are derived from progenitors that are very rare (or nonfunctional) in adult bone marrow; thus, very few B-1a cells are found when bone marrow from adults is used to reconstitute irradiated recipients^{8,16}. B-1b cells, in contrast, are derived from progenitors that are present in adult bone marrow and function readily to reconstitute a B-1b population in irradiated recipients that is roughly half the size of the B-1b population in unmanipulated animals⁸.

Data from feedback regulation studies indicate that the B-1b progenitors detected in adult bone marrow are inactive in intact adult animals; however, they function efficiently to replenish the B-1 population under conditions where feedback regulation is suspended, for example in intact animals in which the B-1 population has been depleted by neonatal anti-IgM antibody treatment^{13,14}. In fact, although B-1b cells tend to be a minor component of the B-1 population in nonmanipulated animals, they are far better represented in the 'recovered' B-1 population than B-1a cells, which are very poorly replenished under these conditions. Taken together, these data suggest that B-1b cells belong to a lineage that is distinct from both the conventional and B-1a lineages.

B-cell progenitor differences

The progenitors for B-1 cells are distinguishable from progenitors for conventional B cells early in ontogeny. Fetal omentum, for example, reconstitutes B-1 cells but fails to reconstitute conventional B cells¹². Similarly, 14-day fetal liver specifically fails to reconstitute conventional B cells in some recipients, even though it generally reconstitutes both B-1 and conventional B cells⁸. Finally, sorted fetal pro-B cells, which have undergone DJ but not VDJ rearrangement, selectively give rise to B-1 cells in both SCID recipients and stromal cultures¹⁸.

Table 1. Milestones in the development of B-1 and conventional B cell populations

Age	B-1 Cells	Conventional B Cells
Fetal	Progenitors HSC appear in liver and omentum (12-13 days); pro-B cells appear and <i>de novo</i> B-1 development begins (16 days).	Progenitors appear in the liver (not in omentum).
Postnatal	<i>De novo</i> development continues; peritoneal population approaches adult size (0-4 weeks).	<i>De novo</i> differentiation begins; conventional B cell populations appear in spleen and lymph nodes.
Adolescent	<i>De novo</i> development terminates; feedback inhibition mechanism blocks new development from progenitors (4-8 weeks).	<i>De novo</i> differentiation continues; populations approach adult levels.
Adult	Individual B-cell clones expand or are deleted (8-20 weeks); hyperplastic and neoplastic (B-CLL) clones appear (>20 weeks).	<i>De novo</i> differentiation continues; populations reach maximal levels (12-14 weeks).

HSC: haemopoietic stem cells

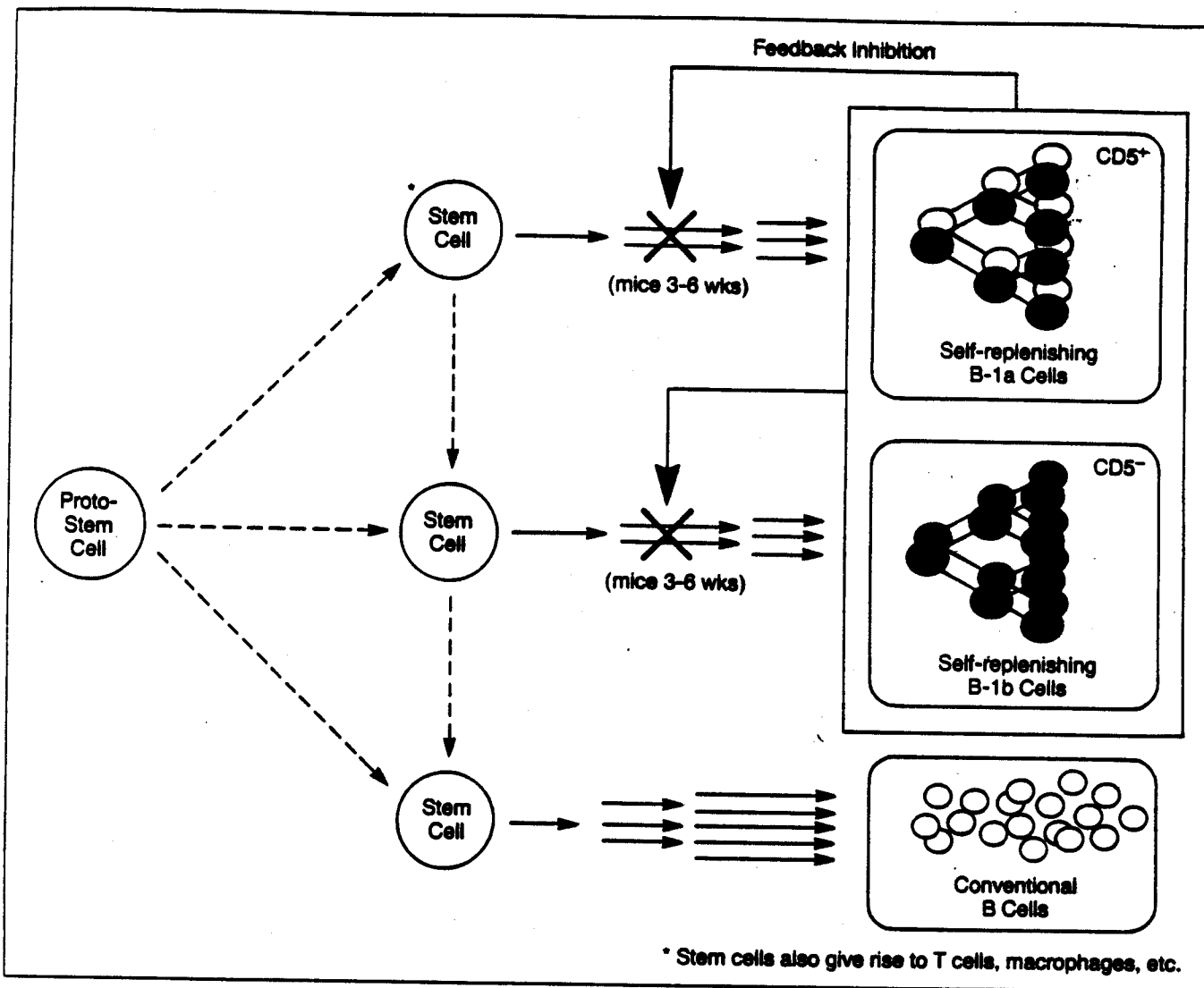


Fig. 1. Development and feedback regulation of B cell lineages. The studies discussed here demonstrate that B-1a and conventional B cells have distinct progenitors ('pro-B cells' or earlier in development) and hence are distinct lineages. As indicated by the dashed lines, it is still unknown when the earliest progenitors (stem cells) diverge. Bone marrow transfers also suggest that B-1b cells constitute a distinct lineage. Feedback inhibition regulates the de novo production of both B-1a and B1b cells and limits new development in the adult.

Later in development, progenitor sources from adult animals (B220⁻ bone marrow or spleen^{8,19}; enriched HSC from bone marrow^{9,10}) fully reconstitute conventional B cells, partially reconstitute B-1b cells, and essentially fail to reconstitute B-1a cells. Furthermore, sorted pro-B cells from adult bone marrow give rise to conventional B cells when transferred into SCID mice or cultured on stromal layers^{19,20}. Thus, both reconstitution and cell culture studies distinguish the progenitors that give rise to B-1 cells from those that give rise to conventional B cells.

The confirmation of this distinction, however, required one further set of experiments. In essence, the evaluation of progenitor capability in transfer recipients was potentially flawed because each source was allowed to condition its own developmental environment. Therefore, we cotransferred fetal liver and adult bone marrow to irradiated recipients and compared the reconstituted B-cell populations to those obtained when the sources were independently transferred⁸. The

results of these cotransfer studies unequivocally demonstrated that the commitment to develop into particular B-cell populations resides with the progenitors themselves rather than with any cotransferred factors that condition the developmental environment. Each progenitor source gives rise to equivalent B-cell populations when transferred alone or together with the other source; neither source influences the development of progenitors from the other. Thus, the differences observed in B-cell populations reconstituted by progenitors from various sources reflect genuine differences in the developmental potential (lineage commitments) of the progenitors present in the source.

Conflicting data and alternative hypotheses

Important questions concerning the origins of B-1 cells have been raised by *in vitro* studies²¹. These were initially interpreted as indicating that CD5⁺ B cells are derived solely from conventional B cells by a particular

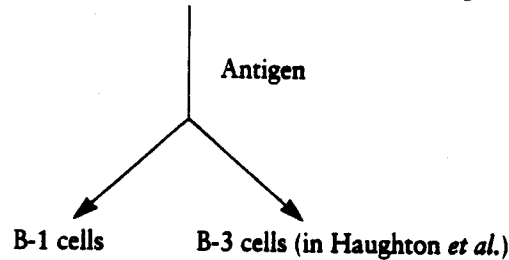
Box 1. Murine B Cells

B-1 cells are localized to the peritoneal and pleural cavities and are found at lower frequency in the spleen. They can be subdivided into B-1a and B-1b cells.

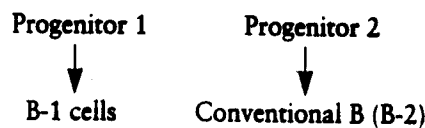
Conventional B (B-2) cells are the predominant type of B cell in the spleen and lymph nodes.

Single Lineage Theory – Antigen triggers differentiation of conventional/uncommitted B cells, which are derived from a single progenitor type, into two types of B cells.

Conventional/uncommitted B (B-0 and B-3 in Haughton *et al.*)



Multiple Lineage Theory – Distinct progenitor cells give rise to separate B cell lineages.



type of activation and selection, namely stimulation with T-independent (TI-2) antigens, which triggers conversion to the B-1a phenotype. This 'selection only' hypothesis is inconsistent with data from the reconstitution studies summarized above, which clearly demonstrate distinct progenitors for B-1a and conventional B cells; however, it has recently been revived²² in a form that is still potentially viable. The current version admits the existence of two B-cell lineages. It proposes that the majority of cells in the B-1a population belong to a 'fetal' lineage whose progenitors do not express terminal deoxynucleotide transferase (TDT), the enzyme responsible for inserting the N-region sequences that increase immunoglobulin diversity. According to this hypothesis²², the failure of the fetal lineage to express TDT results in the rearrangement and expression of surface Ig molecules with (essentially) germ-line-encoded variable regions that have remained in the genome because they code for key antibodies. These antibodies, the hypothesis states, recognize TI-2 antigens (or self antigens that mimic TI-2) which, in combination with cytokines, trigger cells to express the B-1a phenotype and thus select them to persist thereafter as the self-replenishing cells that populate the B-1a lineage.

We categorize this hypothesis as 'possible but not probable'¹ for several reasons. There is no doubt that the earliest HSC give rise to B cells that lack N-regions²³⁻²⁷ and, hence, that the adult B-1 population includes at least some such cells. Furthermore, there is ample data demonstrating that antigens – self, TI-2 or otherwise – and anti-idiotypic antibodies can and do create both positive and negative selective forces that shape the repertoire of the B-1 cells that survive into adulthood. However, although this adult B-1 repertoire is far from well explored, the available evidence

indicates that it contains a wide variety of Ig molecules that have classical N-region insertions and thus are derived from cells that have functional TDT activity²⁶.

The B-1 cells producing these N-region-containing antibodies are postulated (in this TDT hypothesis) to derive from typical 'adult lineage' conventional B cells that have been stimulated by TI-2 antigens and, like 'fetal lineage' B cells, have been triggered to assume the phenotype and self-replenishing characteristics of B-1a cells. This explanation of the origin of B-1a cells is possible, provided that the phenotype conversion demonstrated *in vitro*²¹ can occur *in vivo* and that the 'converted' conventional B cells do become self-replenishing; however, it is not very probable. To be consistent with the available data, the number of such converted cells would have to be very small and/or the process of phenotypic conversion would have to be essentially complete within the first 3–6 weeks of life, since there are essentially no new entrants into the B-1a population after this time. Furthermore, although bone marrow fully reconstitutes conventional B cells and partially reconstitutes B-1b cells, it never reconstitutes more than a few B-1a cells, even in recipients tested many months after transfer.

Other problems with this hypothesis include evidence that the antibody reactivities that have been selected into the B-1 population are not restricted to those that recognize or respond to any particular group of antigens. These cells do make a variety of TI-2 responses; however, they also make T-dependent responses, for example to the phosphoryl choline (PC) hapten presented on protein carriers^{28,29}. Again, further studies are required to obtain a more complete view of the functional repertoire of the B-1a (and other B-cell) populations. However, current evidence is not consist-

ent with the restrictions falling along T-independent or T-dependent lines.

Additional assumptions can be introduced to make the lineage hypothesis conform to the current data. However, it is questionable whether the development of such an unconventional hypothesis is worthwhile: the same overall developmental potential would be assigned to two hematopoietic lineages (fetal and adult) and the functional and phenotypic differences in the B cells obtained from these lineages would be explained by differences in the specificity of the antibodies these B cells produce. Although this is possible, it is more likely that once two developmental lineages have evolved they will continue to diverge and to develop distinctive, heritable phenotypes that fill particular evolutionary niches.

Hematopoietic stem cell lineages

The above considerations suggest that evolution has resulted in the emergence of a series of HSC, the progeny of which create functionally distinct layers within the immune system³⁰. The existence of such layers is supported by the B-cell data discussed here and by data from erythroid and T-cell differentiation studies, which also suggest the successive appearance of functionally distinct HSC during ontogeny. That is, the shift from fetal to adult hemoglobin production in sheep reflects a precisely timed shift in HSC potential³¹⁻³⁴; and the earliest T cells to appear in the mouse fetal thymus (V γ 3) are derived from fetal HSC that are missing or nonfunctional in adult bone marrow^{35,36}. Thus a body of knowledge now exists indicating that HSC either differentiate into, or are replaced by, more mature forms at particular points in development.

The current need to enlarge the older paradigm to allow for distinct HSC that function at different times during development to generate related but distinct lymphoid (and other) subsets is not particularly surprising, given that the concept of a single, unchanging HSC was developed well before technical advances enabled the recognition of different subsets/lineages of B and T cells. Nevertheless, there is a natural reluctance to take this step until all other avenues have been explored. We approve of this conservatism; however, we feel that the current state of the evidence is such that more will be gained by accepting the multilineage origins of lymphocyte subsets and by building upon this concept rather than by looking for ways to discard it. Ultimately, we can expect that this paradigm will also require replacement, as will the next after it, as we continue to bring our ideas about the immune system into agreement with what we have learned.

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they are B-0 cells. This system is likely to work most cleanly when the antigen cannot be recognized by T cells, for example in athymic nude mice, in neonatally thymectomized mice, in T-cell tolerant mice, and when the antigen is not associated with a biodegradable carrier moiety that can be processed by antigen presenting cells. These antibodies are probably not encoded by evolutionarily conserved genes, their specificity does not depend on germ-line encoded CDR3H and, hence, is not disrupted by N region addition. Thus, the ability to produce such antibodies does not decline with age. We find that the antibody response to polyvinyl pyrrolidone (PVP) has these characteristics (Whitmore, A.C., Haughton, G. and Arnold, L.W., unpublished).

According to this hypothesis, the B-1 population may be considered to contain cells producing three distinct, and potentially recognizable, types of antibody. Each of these types is associated with a different function. First, antibody monoreactive with multivalent self antigens, such as anti-PtC. These antibodies are beneficial, are encoded by conserved germ-line genes and their antigen reactivity always involves CDR3H. They are produced, predominantly, by cells whose Ig genes were rearranged early in life. This set may also include the regulatory anti-idiotypes produced by fetal B cells¹⁹ and the 7183.1 (81X) gene product²⁰. Second, antibody that is multi-reactive with a variety of self and non-self antigens. These include, but need not be confined to, 'rogue' antibodies that would subvert the clonal selection process if allowed to interact with T_H cells and develop as B-3 cells. A function of the B-1 pathway in this instance is to remove potentially dangerous cells from the T-dependent repertoire. N regions, of any size, may occur in these cells. Third, antibody that is monoreactive with nonself, multi-valent antigens. These are made by the only B-1 cells responsive to deliberate immunization of the adult; they do not acquire the B-1 phenotype until after immunization and are usually generated from adult bone marrow precursors. Their function is to protect against pathogens by responding to polymeric antigens, such as carbohydrates, that cannot be processed for MHC-restricted recognition by T_H cells.

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Comment by Lee Herzenberg and Aaron Kantor

Haughton and co-workers have presented an interesting and thoughtful extension of a model originally proposed by the Wortis group¹. However, current data seriously challenge the validity of this model.

The Haughton model makes the striking prediction that antibody forming cells generated *in vivo* by TI-2 antigens must express CD5. This is not the case. We have shown that in Balb/c mice stimulated with the TI-2 (thymus-independent, type 2) antigen TNP-Ficoll, less than 5% of the anti-TNP plaque forming cells (PFC) are contained within the sorted CD5⁺ B-cell population². The absence of CD5 on these PFC could be explained if CD5 were routinely lost on B-1a-

derived PFC; however, similar sorting studies with PFC generated in typical B-1 responses, for example to the PC hapten^{3,4} or to the phosphatidylcholine (PtC) determinant on mouse erythrocytes², demonstrate that CD5 is readily detectable on the majority of PFC in these responses. Thus, the recovery of the TNP-Ficoll response in the CD5⁺ population² directly and unequivocally demonstrates that TI-2 stimulation *in vivo* does not induce the expression of CD5.

Even this single example of a failure to find CD5 on PFC following TI-2 stimulation effectively erodes the foundation of the Haughton model. However, this model is also inconsistent with data (reviewed in Ref. 5)

showing that B-1 cells respond well both to α 1-3 dextran⁶ and to the well-known thymus dependent (TD) antigen PC-KLH^{4,7,8}; but they do not respond well to other typical haptens (TNP and NP) coupled either to Ficoll^{2,4,9} or to protein carriers^{2,4,10}. Thus, there is no compelling reason to believe that responses by B-1 (or other B cells) are determined solely by whether the antigen is presented in TD or TI form. Quite the contrary: the prevailing evidence suggests that selectivity of the responses mounted by B-1 and conventional B cells is epitope-based.

Given this varied response pattern, it is difficult to see why Haughton and colleagues claim that response to a TI-2 antigen such as PVP demonstrates that the responding cells are B-1 cells. They cite unpublished data in support of their argument that PVP stimulation leads conventional B cells to differentiate to the B-1a phenotype. However, in view of evidence cited above, demonstrating that stimulation with TNP-Ficoll does not induce CD5 expression on the responding cells, this claim will remain controversial until satisfactory FACS data is reviewed and published.

The N-region sequence argument that Wortis^{11,12}, Haughton and colleagues make is more attractive; however, the representation of N-region sequences in Ig produced by B-1 cells in animals over 4 weeks of age is substantially higher than would be predicted from the observed frequency of new entrants into the B-1 population (Ref. 13 and R. Hardy, this debate)^{5,14,15}. Furthermore, the argument that the lack of these sequences results in the production of germ-line encoded V genes that are more likely to recognize self antigens and be stimulated to differentiate to B-1 cells ignores a substantial body of evidence indicating that a likely consequence of crosslinking slg in the absence of cognate interaction with T_H cells is negative selection, either deletion^{16,17} or anergy^{18,19}. Thus, while the hypotheses advanced by Haughton, Wortis and their co-workers are imaginative and stimulating, the multiple B-

cell lineage hypothesis that we have proposed provides a better and more consistent explanation for current data.

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Comment by Geoffrey Haughton and colleagues

The central issue is whether the B-1 phenotype is determined before cell surface expression of IgM, as a result of some prior commitment in one or more distinct lineages of precursor cell, or whether it is induced by crosslinking of expressed IgM by TI-2 antigen. The difference between the two hypothesis is not trivial, but reflects radically different views of the process of B-cell differentiation.

We do not find the data cited in support of the lineage hypothesis to be compelling. They are also compatible with the notion that the majority of B cells, which Herzenberg and Kantor call B-2 and we prefer to call B-0, are virgin cells that have not yet encountered antigen and which retain the capacity to become either B-1 cells, following a crosslinking interaction or conventional memory cells, following a cognate interaction with antigen and T_H cells. The cell transfer data relate only to B-1 cells that arise spontaneously, without delib-

erate immunization. They overlook the possibility that these cells could be responding to self antigens which, of course, would confer the capacity for self-renewal for so long as the antigen remained available in the animal, and would result in the selection of a set of cells expressing a selected repertoire of V genes, precisely as found.

It is undeniably true that a greater proportion of B cells generated from fetal liver than from adult bone marrow, spontaneously assume the B-1 phenotype. We view this as evidence of maturation of a single developmental lineage of B cells, rather than as evidence that multiple lineages may co-exist. We have offered an explanation of how this change might come about. However, we do not suggest that B-1 cells can only be generated from fetal precursors, nor that Ig gene rearrangements of B-1 cells are necessarily devoid of N nucleotides; any B cell that expresses antibody that can be crosslinked by antigen could be induced to assume