CORRESPONDENCE

ESTIMATION OF HEMATOPOIETIC CLONE NUMBERS

To the Editor:

In their recent paper, Harrison et al attempt to show, by using data from murine embryo aggregation chimeras, that "many, or even all of the primitive stem cells may simultaneously contribute to erythropoiesis." More precisely, they state that "the average of the numbers of primitive stem cells whose progeny actively produced reticulocyte clones during the three-day reticulocyte lifespan... was 64 (range, 33 to 118). This is far too high for such cells to be serially activated and inactivated, as required by clonal selection hypotheses."

The validity of the numbers given and of the conclusions drawn both rest, we believe, on assumptions that are unlikely to be correct. We suggest that the data that Harrison et al present probably throw no light on the constitution or behavior of any early hematopoietic stem cell compartment.

Clone numbers were estimated from the variance of the proportion of one of two types of hemoglobin (Hb) measured in serial blood samples. Although the authors tried to take experimental error into account, their estimate of the error was based on the variance calculated from normal F1 hybrid mice (in which the ratio of the two Hb types was near 1:1), whereas in the chimeras themselves the ratio was reported to be about 1:9. The experimental error cannot, however, be assumed to be independent of the mean proportions (p and q) of the two Hb types. The issue is further complicated by the fact that 4.4% - 4.6% of d-type Hb was measured experimentally in samples that were known to contain none in reality. Thus there must be considerable uncertainty about the real degree of chimerism when (as in most of the animals described) the measured proportion of d-type Hb was only in the 7% to 12% range: this will affect the values of both p and q used in the binomial equation for calculating clone numbers. It must also be noted that large numbers of animals are needed to achieve reasonably narrow confidence limits for variance estimations; such numbers are clearly impracticable for studies on embryo aggregation chimeras, so wide confidence limits have to be accepted.

Of the other assumptions that are required to estimate clone numbers when the binomial equation is used, two are suspect on the basis of the authors' data. (a) The first assumption is that the proportion of CBA cells (as represented by d-type Hb) in the sample is the proportion of CBA clones that constitute the population. Although Harrison et al explicitly make this assumption, it seems to be contradicted by the evidence that "CBA cells did not grow as well as B6 cells in the erythroid lineage..." This latter statement was based on the fact that, according to coat color mosaicism, CBA and B6 were approximately equal contributors to the chimeras, whereas B6 predominated in the erythrocytes. Although this imbalance could have occurred solely in embryogenesis before establishment of the adult stem cell pool, the reported discrepancy between lymphocytes and erythrocytes suggests that there was selection against the CBA phenotype in erythropoiesis at some post-stem cell stage(s). (b) The second assumption is that serial measurements are independent in the sense that no clone (identified as a product of binomial sampling) contributes to more than one measurement. The authors conclude on the basis of serial correlation analysis that the samples were indeed independent. However, if (as Harrison et al say) much of the measured variance was due to experimental error, this (presumably uncorrelated) variability could obscure an existing dependence between successive sample values.

There are several reasons, therefore, for believing that the estimates of clone numbers in these mice may not be even approximately correct. It seems that biologic variation between serial chimeric blood cell samples is so small, at least in the short term, that highly optimized experimental models will be needed to measure it.

Nevertheless, the interpretation of data of this kind remains a matter of interest. Taking at face value the estimate of 64 "primitive stem cells whose progeny actively produced reticulocyte clones" over three days, we are puzzled as to why this should be considered a high number or one that is incompatible with clonal succession. Although no attempt was made to define the meaning of "primitive stem cell," a very small compartment of no more than a few hundred cells seems to be implied. This may be compared with a pool of spleen colony-forming cells (CFU-S) of about 500,000. Perhaps only a small proportion of CFU-S are stem cells, so a more instructive comparison might be with cells capable of effecting long-term repopulation of genetically anemic or lethally irradiated recipients. The frequency of these is estimated to be about one in 50,000 nucleated bone marrow cells, which implies a pool size of some 10,000. On Harrison et al's figures, less than 1% of them would have to produce differentiating progeny over a three-day period to fuel erythropoiesis, allowing ample scope for clonal succession.

Harrison et al suggested the possibility that the biologic variation was too small to be detected against the background noise and hence that the number of clones present was indeterminately large. If this is true, it might appear to constitute stronger evidence against clonal succession. We would argue that it does not. Everything depends on what, in the context of these experiments, can be understood by the term clone. Most simply, a clone can be defined as any group of cells that share a common precursor or "founder" cell. The founder can be any cell that is capable of division. The size of such a clone at any point in time will be limited by the number of cell generations that have passed since its foundation. If one wishes to use a binomial approach to estimate clone numbers by exploiting genetic markers such as Hb, it is necessary for the founders to be drawn at random from a pool of potential founders. In chimeras carrying Hb markers, the variance of serial measurements may give some indication of the number of erythroid clones, founded by members of such a precursor population, that have disappeared and been replaced during the interval between measurements. This, in turn, allows some inferences to be drawn about the average size of the clones and hence whether they are derived from ancestors that were a few or many generations distant. Harrison et al assume that the precursor pool from which clonal founders are drawn is necessarily the pool of primitive stem cells. This assumption is unproved and indeed imprecise. It ignores the possibilities (a) that the stem cell compartment is itself "structured" and (b) that reserves of precursor cells may exist at later stages of hematopoiesis (for example, erythropoiesis-sensitive cells [ESC]). The rapidity of the erythropoietic response to anemia or administered erythropoietin and particularly the evidence that ESC continue to cycle even in long-term polycythemic mice suggest the existence of such reserves. If they exist, there will be more than one stage of hematopoiesis at which a limited number of founder cells may be drawn from a larger pool of potential precursors. It may then be difficult to decide which stage particular experimental data are telling us about. This stage is likely to depend on the interval between successive measurements. For example, the binomial variance between serial measurements of reticulocyte Hb made at three-day intervals may be the result of sampling from the ESC pool; in that case it will provide information about the number of clonogenic ESC but no information about the numbers of clones.
initiated at earlier stages of hematopoiesis, eg, by stem cells. Conversely, measurements made at intervals of weeks or months will skip over any short-term fluctuations in the chimerism of the sis (involving, presumably, fewer but larger clones).

The foregoing argument predicts that binomial variability will tend to increase with increasing time between measurements. This is what Harrison et al found, but they considered the result paradoxical; it must, they reasoned, have been due to experimental error because one could not have fewer primitive stem cells activated to form clones over a 45-day than over a 3-day time span. The paradox vanishes if it is accepted that “clones” can be initiated at different stages of the hematopoietic hierarchy.

A final point concerns terminology. Clonal succession was introduced by Kay and has been used since by others. More recently, in Harrison's paper and elsewhere, the variant clonal selection has begun to creep in. The latter term was introduced long ago by Burnet to describe the selective activation of specific lymphocytes by an antigen. It expresses one of the fundamental concepts of immunology. There seems to be no reason for the neighboring field of hematology to adopt the same term for a quite different process.

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REFERENCES


To the Editor:

The comments of Micklem and Gray on our article (Blood 69:773, 1987) afford a valuable opportunity to clarify some points about how the most primitive hematopoietic stem cells (PSC) replicate and about the use of the binomial formula. Our purpose, as stated in the abstract and introduction, was to test the hypothesis that erythropoiesis results from clonal succession—the differentiation of one or a very small number of PSC that are sequentially activated to proliferate and form clones of differentiated cells and then eventually decline and be replaced by new stem cell clones. This model predicts large variations within each individual in the proportion of hemoglobin made by each of their two distinguishable PSC types, especially in newly synthesized erythrocytes. Our data clearly contradicted this model with only small variations, both in newly synthesized erythrocytes tested at short (three- to eight-day) intervals and in the total erythrocyte population tested at long (2- to 4-month) intervals. We concluded that PSC responsible for long-term erythropoiesis in unmanipulated individuals do not differentiate a few at a time in terminal bursts and suggested an alternate hypothesis—that PSC function continuously. To this degree our data throw light on the behavior of the PSC compartment, contrary to the assertion of Micklem and Gray.

Most of their letter disputes our estimates of PSC numbers. Much of this dispute seems unnecessary. We stated throughout the paper that our numbers were underestimates because our calculations did not exclude the contributions of experimental error to the overall variability. However, the critical issue was whether there was only one or a very few PSC activated every three to eight days (Table 1) or every 60 to 136 days (Table 3), and our data strongly contradicted this.

Equally important is the evidence that PSC may be continuously active. Although the values in Table 1 were taken over only 47 days whereas those in Table 3 were taken over 700 days, they are similar, with the absolute mean ± SD value of their difference only 2.3% ± 2.2%. Thus, essentially constant proportions of each hemoglobin type were produced over the entire life span of each chimeric mouse. This could be produced by constant activation and inactivation of large numbers of randomly sampled precursors, but it also could be produced by any of a wide range of PSC numbers if the same PSC were continuously active and producing constant proportions of hemoglobin lifelong. In the latter case, successive blood samples from the same mice would not be independent; thus, calculations using the binomial formula would not be meaningful, and numbers of active PSC might not be large, contrary to our article.

Micklem and Gray's calculations based on 64 PSC do not take this into account, nor do they consider that our numbers gave only minimum values. Their suggestion that reserves of differentiated precursor cells may contribute to erythropoiesis and allow short-term measurements to reflect a larger pool than long-term measures may be correct but seems unnecessarily complex. The most simple model of erythropoiesis with structured precursor compartments is that the proliferative capacity is lower when the cell is more differentiated. This is supported by common observations that less primitive precursors produce smaller colonies in vitro and in vivo. Furthermore, cycling studies generally find higher proportions of cycling cells in more differentiated cell populations, which causes, for example, their well-known greater vulnerability to 5-fluorouracil.
Estimation of hematopoietic clone numbers [letter]

HS Micklem and RA Gray