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Clonal fluctuation within the haematopoietic system of mice reconstituted with retrovirus-infected stem cells

Ralph Snodgrass and Gordon Keller

Basel Institute for Immunology, Grenzacherstrasse 487, CH-4058 Basel, Switzerland

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The clonal make-up of the haematopoietic system of mice reconstituted with retrovirus-infected bone marrow cells was analysed at two different points in time following reconstitution. We have found that under these conditions, the haematopoietic system consists of clones that persist throughout the 5 month course of the experiment as well as those which undergo temporal changes. The various changes that we have observed included the appearance of a new clone(s) in all lineages, the loss of a clone from some lineages and the shift in the appearance of a clone from one lineage to another. In addition, we provide evidence which suggests that the clonal make-up of the thymus changes with time; early after reconstitution it consists of many clones, whereas at the later time-points it contains a limited number of predominant clones. These studies document the dramatic clonal changes which occur within the various lineages for a long time following reconstitution and highlight the difficulty in demonstrating lineage-specific stem cells.

Key words: haematopoiesis/retrovirus/stem cells/thymus

Introduction

Experiments utilizing unique cell markers, either radiation-induced chromosomal translocations (Wu *et al.*, 1968; Edwards *et al.*, 1970; Abramson *et al.*, 1977) or retroviral integration sites (Dick *et al.*, 1985; Keller *et al.*, 1985; Lemischka *et al.*, 1986; Keller and Wagner, 1986), have demonstrated that all haematopoietic lineages (lymphoid and myeloid) arise from a common precursor, the multipotent stem cell. In addition to their multi-potentiality, stem cells also exhibit substantial self-renewal capacity, thereby enabling them to function *in vivo* over significant periods of time. It is not known whether these stem cells are truly 'immortal', functioning for the entire life of an animal, or whether they have a limited life span. If stem cells have a limited life span, then only a portion of the total stem cell pool would be active at any given time, with new ones becoming active as old ones die. The haematopoietic system would then be maintained by a succession of relatively short-lived clones. If this is the case, it is important to know the life span of the clones and to what extent these clonal changes are found within the individual haematopoietic lineages. Such change would have important implications for cells of the lymphoid system, particularly with respect to repertoire generation and tolerance induction. A number of earlier studies have suggested that the clonal make-up of the haematopoietic system can change with time (Micklem and Loutit, 1966; Micklem *et al.*, 1983; Mintz *et al.*, 1984; Lemischka *et al.*, 1986). However, none of these studies analysed the clonal make-up of individual haematopoietic lineages within the same animal at different points in time.

We have examined, over a 5 month period, the clonal make-up of the various haematopoietic lineages including the thymus of mice reconstituted with retrovirus-infected bone marrow cells and report here that (under these conditions) the haematopoietic system consists of a combination of stable clones which show no change, as well as clones which undergo dramatic temporal changes during the course of the experiment. In addition, we provide evidence indicating that a multipotent stem cell may produce progeny which contribute predominantly to a limited number of lineages at one time and then to other lineages at another time. Finally, we present data from animals with thymic grafts which indicate that early after reconstitution, the thymus contains many clones whereas at later times it consists of one or two predominant clones.

Results

Experimental approach

Our experimental protocol is presented in Figure 1. Mice were repopulated with a common pool of bone marrow cells which

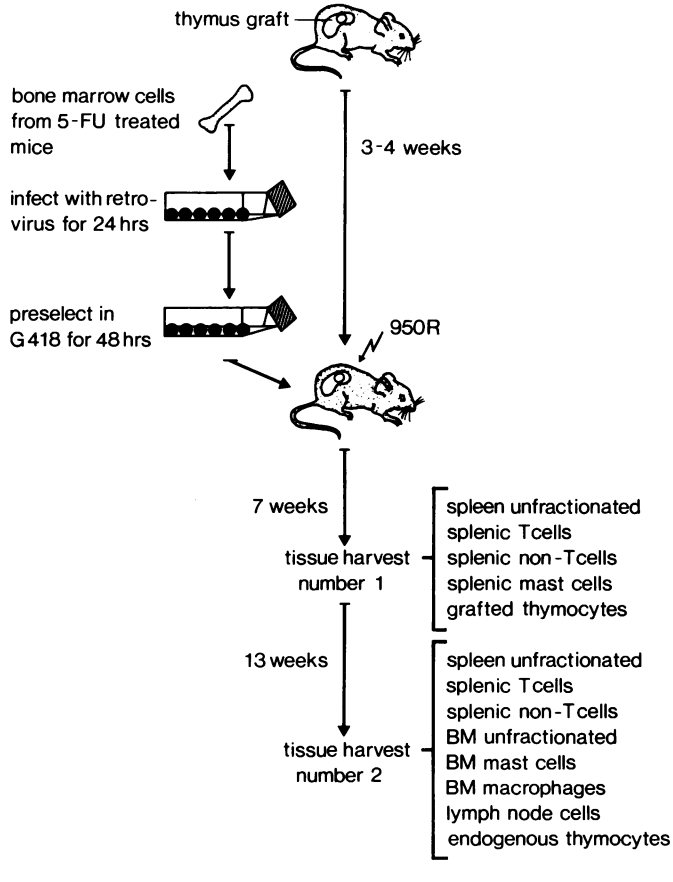

Fig. 1. Diagram of experimental protocol. CBA/N bii (12 week old) mice were grafted under the kidney capsule with syngeneic 16–17 day embryonic thymic lobes. Three to four weeks later, the mice were irradiated (950 R) and reconsituted with bone marrow (CBA-H T6T6, Bantin and Kingman) which had been infected with a defective retrovirus as described previously (Keller *et al.*, 1985). At 7 and 20 weeks following reconstitution, tissue samples were taken and various cell subpopulations were isolated. See Materials and methods for details.

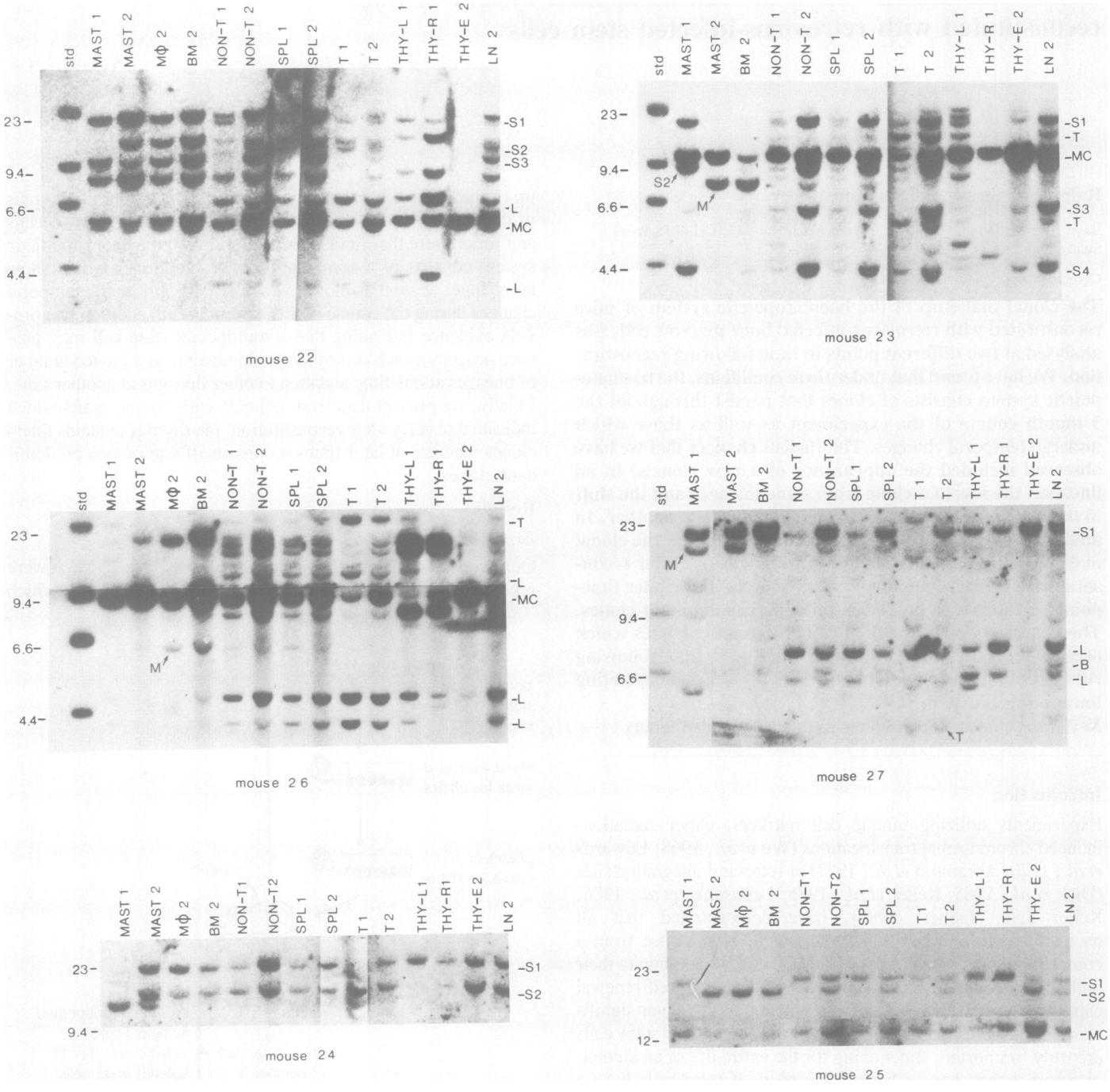

Fig. 2. Clonal analysis of various haematopoietic lineages at two time points following reconstitution. Southern blot analysis was performed with DNA from cell populations (see Materials and methods for details on cell separations) from six recipients prepared at 7 weeks ('1') and 20 weeks ('2') following reconstitution. The blots were hybridized by standard techniques with a probe containing the coding region of the bacterial *neo* gene from Tn*5*. The left hand track contains mol. wt standards. On the right side are clone designations: MC refers to a major clone derived from multipotent stem cells found in all lineages at both time points; S refers to clones derived from multipotent stem cells not present in all lineages or all tissues at both time points; M refers to myeloid clone; L refers to lymphoid clone; T refers to T cell clone; and B refers to B cell clone. Above each track is an abbreviation to indicate the cell population from which the DNA was isolated; i.e. MAST, mast cells; MΦ, macrophage; BM, bone marrow; NON-T, splenocytes depleted of T cells; SPL, unfractionated splenocytes; T, splenic derived Con A blasts; THY-L, THY-R and THY-E thymic grafts under the left and right kidney capsule and the endogenous thymus respectively; and LN, lymph node.

had been clonally marked by retroviral integration sites. To gain access to thymic tissue at different time points, the recipients were given thymic grafts prior to irradiation and reconstitution. Tissue samples were taken at 7 and 20 weeks following reconstitution and cell populations representing various lineages were prepared. The clonal composition of these populations was analysed by Southern blots with probes specific for the transducing retrovirus. Due to limiting amounts of material, the B lymphocyte compartment was analysed indirectly by comparing a splenic population devoid of T lymphocytes (~75% B cell, 25% myeloid cells) to myeloid populations represented by mast cells. Any major change which occurred in the splenic non-T population but not

in the myeloid population was attributed to the B lymphocyte lineage.

Clonal changes with time

Figure 2 shows the complete analysis of six different recipients (mice 22–27). These animals were reconstituted with large numbers of donor cells (see Materials and methods) to maximize the number of stem cells capable of participating in the reconstitution. In almost all of the animals we could identify clones that changed with time as well as those which were stably expressed throughout the 20 week course of the experiment (Figure 2). The most striking examples of this latter category were found in mice 22, 23 and 26 (Figure 2, marked MC, indicating a Major Clone). In each mouse this clone was a dominant clone in all tissues at both time points and was the major, if not the only, one found in the endogenous thymus. The fact that these clones included both myeloid and lymphoid cells and persisted for 20 weeks, indicated that they were derived from multipotent stem cells. It is, of course, possible that these clones would have undergone some changes if the experiment had been carried out for a longer period of time. We have recently analysed a second group of mice and have identified clones that were stably expressed for one year (data not shown). Thus, it appears as if some stem cells can function for a significant part of, or perhaps for the entire life of an animal.

Among the clones which showed changes, we observed a variety of different patterns (summarized in Table I). One change was the appearance of a new clone in all lineages and tissues at the second time point (Figure 2, mouse 25 'S2', indicating a stem cell-derived clone). This type of pattern strongly suggests that all of the multipotent stem cells are not active at the same time. A second category of change was a quantitative change in the expression of a clone in different lineages at the two time points. Within this category, we have identified situations in which a particular clone was expressed predominantly in a myeloid lineage at the first time point and then in the lymphoid lineages at the second time point. The most striking example of a myeloid to lymphoid switch was found in mouse 23 (Figure 2, 'S1, S2 and S4'). At the first time point these clones (these bands may represent one clone with three integrations or three discrete clones) were present in the mast cell population but contributed little, if at all, to the lymphoid lineages. This situation was reversed in the second analysis where they were only found in the lymphoid lineages. Similarly, in mouse 22 the clone marked 'S3' was present predominantly in the mast cells at the first time point and then in all lineages at the second time point. At that time, the frequency of this clone was much greater in the splenic non-T and the lymph node populations than in the purified T cells, suggesting that it contributed more to the B- than the T-cell lineage. A variation of this pattern was found in mouse 27. At the first analysis, the clone marked 'S1' was present in the mast cells as well as in the grafted thymuses. However, it was not detectable in the peripheral lymphoid population. By the time of the second analysis, it was a major clone in both lymphoid lineages. The converse pattern, i.e. found early in the lymphoid and later in the myeloid lineages, is also seen. In mouse 24 the clone marked 'S2' was found in the splenic non-T cells as well as in T cells, but not in the mast cells at the first time point. At the second time point this clone was found in all lineages. A similar pattern is seen with the 'S2' clone in mouse 22. Finally, we have observed one clone (S1, mouse 25) that was present in all lineages at the first time point, but could be detected only in the lymphoid populations at the later time point.

The retroviral vector, N2, used in these studies is replication defective (Keller *et al.*, 1985) and viral stocks were produced with the ψ-2 'helper-free' packaging cell line (Mann *et al.*, 1983). However, it is known that ψ-2 can transfer packaging competence at a low frequency and can produce helper virus presumably by recombination (Mann and Baltimore, 1985; Miller and Buttimore, 1986; Miller *et al.*, 1986). To determine whether or not any of our recipients contained infectious virus, we analysed their sera for the presence of virus capable of transducing G418 resistance. Indeed, we found low levels of virus in the sera of most of our recipients (mouse 22, 2700/ml; mouse 23, 1600/ml; mouse 24, 300/ml; mouse 25, none detected i.e. <100/ml; mouse 26, 4300/ml; and mouse 27, 700/ml). Viral spread may complicate the interpretation of clonal changes in those situations where new clones are detected at a later time point. In our analysis, this presents a problem only with clone S2 in mouse 25. However, since this animal contained no detectable virus, it is unlikely that the appearance of this clone was due to a secondary infection.

Lineage-restricted clones

Most of the reconstituted animals contained some clones which were found in a limited number of lineages at the two time points. Four mice (22, 23, 26 and 27) had clones which were expressed predominantly in the lymphoid lineages at both assay times; either in both T and B cells (Figure 2, 'L'), only in T cells (Figure 2, 'T') or only in B cells (Figure 2, 'B'). Three mice (23, 26 and 27) had clones which were only detectable in the myeloid lineage (Figure 2, 'M'). It should be stressed that these clones were detectable in only these lineages at two time points 13 weeks apart.

There are many examples where the splenic-derived mast cells isolated at the first time point were clonally different from the bone marrow-derived mast cells isolated at the second time point. This is not due to the different source of precursor cells since mast cells derived from the bone marrow and the spleen at the same time point are clonally identical in most recipients (Figure 3a).

Thymus repopulation

Several reports have suggested that the thymus is colonized by very few, perhaps one, cell(s) (Wallis *et al.*, 1975; Kadish and Basch, 1976; Ezine *et al.*, 1984). In all six recipients the endogenous thymus, analysed at 20 weeks, consisted of a very limited number of clones which were derived from multipotent stem cells, whereas the grafted thymuses analysed at seven weeks, contained additional clones which were more restricted in expression either in time or within the various lineages (Figure 2). One of the unexpected observations was the extent to which the clonal make-up of the grafted thymuses differed from that of the endogenous thymus. The remarkable similarity of the grafted thymuses to the non-lymphoid populations raised the possibility that the grafted thymuses were infiltrated by myeloid cells. Analysis of the T cell receptor β chain gene rearrangements demonstrated that this was not the case. The same filters as in Figure 2 were eluted and rehybridized with the T cell receptor β chain constant region probe. Figure 4 illustrates the results from mouse 23 and is representative of all the mice. In this analysis, the β locus has two germline bands at 9 and 3 kb representing $C_\beta 1$ and $C_\beta 2$ loci, respectively. Any rearrangement (excluding inversions) of these loci results in the depletion of the 9 kb band. The 3 kb band does not rearrange and serves as an internal standard (Snodgrass *et al.*, 1985). To estimate the extent of Ig heavy chain rearrangements, the same filter was again eluted and rehybridized with an immunoglobulin heavy chain $J_H 1$ probe (Figure 4). Any rearrangements of the Ig_H loci results in a depletion

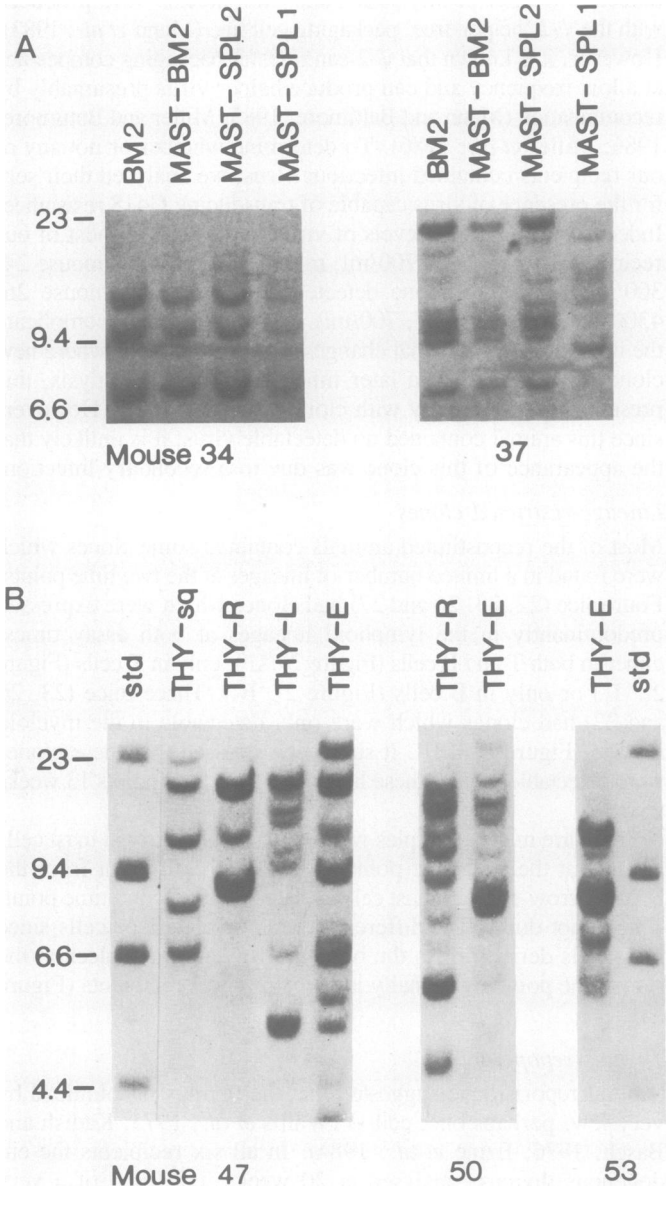

Fig. 3. Clonal analysis of splenic versus bone marrow-derived mast cells and grafted versus endogenous thymuses. **A**, Mice were grafted, reconstituted, and analysed as described in Materials and methods. Mast cells were prepared from both bone marrow and spleen cells at the second time point. **B**, These animals were repopulated with bone marrow cells that had been infected with virus containing supernatant rather than by co-culture. One animal (No.47) received a subcutaneous thymus graft in addition to grafts under the kidney capsule. Nine weeks after reconstitution the clonal make-up of the thymuses was analysed.

of the 2.2 kb J_H1 germline band. Below each track in Figure 4 is an estimate of the extent of rearrangements of the loci within the population analysed based on densitometeric analysis of the autoradiograms. From this analysis it is clear that in the grafted thymuses, as well as in our T-cell preparations, essentially all of the β alleles have been rearranged. This suggests that these populations were >90% T cells. The mast cell populations showed no significant rearrangement at either locus, indicating that they contained very few contaminating lymphoid cells. These analyses confirm the purity of our cell preparations. The peripheral T cells, as well as the thymocyte preparations, have substantial J_H rearrangements. It is known that both of these

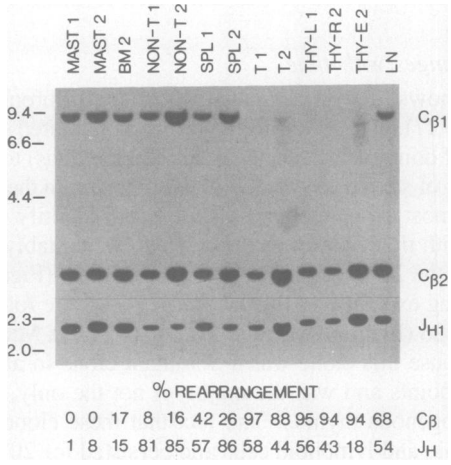

Fig. 4. Extent of rearrangement of the T cell receptor beta chain and the immunoglobulin heavy chain loci. The filter from mouse 23 (Figure 2) was stripped and rehybridized sequentially with a T cell receptor β chain constant region probe and then the immunoglobulin heavy chain J_H1 probe. Below the figure is a calculated estimate of the extent of rearrangement for each population at both loci. See Materials and methods for details.

populations undergo aberrant immunoglobulin heavy chain D−J rearrangements (Cory *et al.*, 1980; Kemp *et al.*, 1980; Kurosawa *et al.*, 1981; Reth and Alt, 1984), but it is intriguing that less J_H rearrangements occurred in the endogenous thymus compared to the grafted thymuses. The significance of this observation is unclear and will require further analysis. The data displayed in Figure 4 also demonstrate that the DNA analysed in our experiments was digested to completion. This is suggested by the absence of a ladder of the $C_\beta1$ and the J_H1 bands which is characteristic of a partial digest.

The simplest explanation for the differences in the clonal make-up between the grafted thymuses and the endogenous thymus is the difference in time of analysis. The grafted thymuses were analysed at week 7 following reconstitution whereas the endogenous thymus was analysed at week 20. To determine whether or not time is a significant factor in the clonal composition of the thymus, we analysed grafted and endogenous thymuses at the same time following reconstitution. The results from three animals indicate that at 9 weeks after reconstitution the endogenous thymus contained many clones and the thymus grafts from two of these animals contained a similar number of clones (Figure 3b).

Discussion

This report is the first analysis of the clonal make-up of the individual haematopoietic lineages at different time points within the same animal. We have identified at least two categories of clones which contribute to the haematopoietic system of a reconstituted mouse; those which are stably expressed for long periods of time and those which undergo dramatic changes (Table I).

A number of other reports have provided evidence suggesting that the haematopoietic system can undergo some clonal fluctuation. Micklem and colleagues (Micklem and Loutit, 1966) followed the fate of uniquely marked clones through sequential transplantations. Although a complete lineage analysis was not done, they did observe that a particular clone could contract or expand when transplanted. We have also observed clonal changes when bone marrow cells, that were marked with a retroviral vector, were serially passaged through irradiated recipients (Keller

Table I. Clonal changes in reconstituted mice

Mouse	Clone	Type of change[a]	
		Myeloid	Lymphoid
22	S2	gain	nc[b]
22	S3	nc	gain
23	S1,S2,S4	loss	gain
24	S2	gain	nc
25	S1	loss	nc
25	S2	gain	gain
27	S1	nc	gain

[a]Change in relative clone size between 7 and 20 weeks.
[b]nc: no change.

et al., 1985). However, in these instances we noted only a loss of clones. More recently, Lemischka *et al.* (1986) have shown that new clones can appear upon transplantation of bone marrow. However, in light of the fact that ψ-2 based vectors are not totally 'helper-virus'-free (see Results), the possibility exists that some of the new clones could have been due to viral spread. In addition, they attempted to follow clonal changes within an individual recipient by analysing samples of peripheral blood at several times after reconstitution. Although some changes were observed, it was not possible to determine in which lineages the changes were occurring, and due to the intensity of the signal, only the most major clones were detectable.

The data presented here indicate that the haematopoietic system of a reconstituted mouse consists of clones which are stably expressed and clones which undergo temporal changes. The differences amongst these clones might reflect differences in the stem cells from which they were derived. One would expect the most primitive stem cells with the highest self-renewal capacity to generate clones that would dominate the haematopoietic system for a long period. The major clones (MC) found in mice 22, 23 and 26 represent the progeny of such primitive stem cells. Once established this type of clone might persist throughout the life of the animal. Indeed it has been previously observed that a single clone derived from a multipotent stem cell can persist from more than 1 year *in vivo* (Keller and Wagner, 1986), and recently in another series of mice, we have observed several examples of clones that have persisted for similar periods of time (unpublished observations). Smaller clones and those clones found in a limited number of lineages, may be derived from more mature stem cells having less self-renewal capacity. These clones would be expected to become extinct with time. Alternatively, it is possible that some of these clonal differences are due to stochastic processes. That is, the first stem cell to colonize the bone marrow may simply have a substantial growth advantage over other equally competent cells. At present, we cannot distinguish between these possibilities.

The persistence of a clone in the different lineages is directly related to the life span of the cells within these lineages. A significant fraction of both B and T cells have a long life span (Sprent and Basten, 1973; Freitas *et al.*, 1986) whereas many of the myeloid cells are relatively short-lived (Cronkite, 1969). Thus, one might expect clones to persist longer in the lymphoid than in the myeloid lineages. This may be one reason why we found clones with an apparent lymphoid restriction (Figure 2; clones marked L, B and T) in most of our recipients. These clones may have contained myeloid cells earlier than the first time point. If these mice would have been analysed at only the later time point, a lymphoid 'restricted' pattern of development would have been observed for the clones marked S1, S2, S4 in mouse 23 and S1 in mouse 25. Yet, these clones were clearly not derived from a restricted cell, since they contained cells of a myeloid lineage at an earlier time. It is possible that some of the lymphoid restricted clones were indeed derived from stem cells that were restricted to the lymphoid lineages. However, our findings that clones derived from multipotent cells can alter their patterns of expression with time, clearly emphasizes the difficulties in demonstrating the existence of restricted stem cells. Two reports have suggested that restricted stem cells do exist. In one study, recipients were analysed at only one point in time (Dick *et al.*, 1985). In the second report, both primary and secondary recipients were analysed (Abramson *et al.*, 1977) and although the same 'restricted' pattern was observed in both recipients it is still possible that the stage at which these clones were expressed in other lineages was missed.

Our data on thymus repopulation indicates that at 20 weeks after reconstitution the endogenous thymus is populated by a limited number of clones. Although this is consistent with several experiments which have concluded that the thymus is colonized by very few cells (Wallis *et al.*, 1975; Kadish and Basch, 1976; Ezine *et al.*, 1984), our findings do not directly address how many cells initially colonize the thymus since it is possible that the thymus is seeded by multiple cells of the same clone. The difference between the grafted thymuses and the endogenous thymus is very striking. Preliminary results indicate that these differences may be due, in part, to differences in the time of analysis. The endogenous thymus was analysed at 20 weeks, whereas the grafted thymuses were analysed at 7 weeks. When grafted and endogenous thymuses were analysed at the same time, both were found to contain multiple clones (Figure 3b). This evidence suggests that the clonal make-up of the thymus changes with time.

There are a small number of clones in the peripheral T cell population which were not found in any of the thymuses. The explanation that we favour is that this represents the temporal changes in the clonal composition of the thymus. It is likely that the additional clones in the periphery are the progeny of precursors which have previously passed through the thymus and have now been replaced by other clones. A possible example of this type of clonal evolution is exemplified by the 'T' clone of mouse 26 (Figure 2). The alternative explanation that we cannot exclude is that some peripheral T cells develop extra-thymically.

A further point of interest with respect to the grafted thymuses is that the majority of the time they were repopulated by identical clones. The extreme example of this is mouse 22 where the grafted thymuses contained six identical clones (Figure 2). Likewise, the three grafted thymuses and the endogenous thymus in mouse 47 contained at least one clone in common. This would suggest that there is substantial expansion of the precursor pool at some stage prior to entry into the thymus and that these cells are available to repopulate the thymus at approximately the same time.

In conclusion, these data indicate that the haematopoietic system of a reconstituted mouse is not maintained entirely by a succession of short-lived clones, but rather consists of clones which are stably expressed for long periods of time as well as those which undergo dramatic temporal changes. It has been possible to demonstrate that the contribution of a multipotent stem cell to the different haematopoietic lineages can vary considerably with time. This fluctuation makes it difficult to identify lineage restricted stem cells and to study precursor/progeny relationships within the haematopoietic system. We also provide evidence indicating that the clonal make-up of the thymus changes

dramatically with time. Understanding these aspects of the development of haematopoietic clones in an irradiated reconstituted animal, in addition to its intrinsic interest, is of clinical relevance for chemotherapy, transplantation, and future gene therapy.

Materials and methods

Bone marrow infection and reconstitution

Each recipient was grafted with day 17 embryo thymuses under the kidney capsule. After 3–4 weeks the animals were reconstituted as described previously (Keller *et al.*, 1985) with 2.6×10^6 infected and pre-selected bone marrow cells which were derived initially from the femurs and tibias of six donors. Greater than 85% of the colony-forming-cells in this population were resistant to G418 (1 mg/ml active substance).

Cell preparation

Seven weeks following reconstitution half of each thymus graft and half of the spleen were removed. Unfortunately, we could not resample the same thymus at a later time point as intended, since in all cases the remainder of the grafted thymus was re-absorbed. Splenic cells were divided into four portions. DNA was prepared from one portion. The second portion, referred to as 'T', was cultured for 7 days in the presence of concanavalin A (5 μg/ml) and IL-2 (rat Con-A supernatants) to yield a population enriched for T cells. The third portion was treated with saturating amounts of monoclonal anti-Thy-1.2 and complement to yield a 'non-T' population. The dead cells were removed from the 'T' and the 'non-T' populations by Lymphoprep density gradients. DNA was prepared from each of these populations. Typically >95% of the T cells and <5% of the non-T cells expressed Thy-1. Furthermore, ~75% of the non-T cells and <5% of the T cells were surface Ig positive. The fourth portion of spleen cells was cultured for >3 weeks in medium containing WEHI-3B supernatant as a source of IL-3. At the end of this culture period, >90% of the cells had the morphology of mast cells with May-Grünwald-Giemsa stain. DNA was prepared from this population and is referred to as 'MAST' in subsequent figures. At 20 weeks after reconstitution the animals were killed and blood samples were collected for virus testing. The remainder of the spleen (SPL), the bone marrow (BM), lymph nodes (LN) and the endogenous thymus (THY-E) were removed. The spleen cells were separated, as above, into the various subpopulations with the exception that mast cells were prepared from bone marrow instead of spleen. In addition, bone marrow-derived macrophages were isolated by culturing for 7 days in the presence of L-cell conditioned medium. DNA was prepared from these eight cell populations.

Southern blot analysis

DNA (5–10 μg) was digested with *Hin*dIII and analysed by the alkaline Southern blot method (Reed and Mann, 1985). The J_H1 probe is the 600-bp *Bam*HI–*Hin*dIII fragment containing only the J_H1 region (Bernard and Gough, 1980; Early *et al.*, 1980). The quantitation was performed by densitometeric analysis of autoradiograms with different exposure times. The intensity of the $C_\beta2$ was used as an internal standard to take into account the amount of DNA loaded. The values presented are the averages from mice 22, 23, 26 and are based upon depletion of the germline bands of $C_\beta1$ and J_H1, using the $C_\beta2$ band as a standard.

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