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Acquired skewing of X-chromosome inactivation patterns in myeloid cells of the elderly suggests stochastic clonal loss with age

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Summary. More frequent skewing of X-chromosome inactivation patterns (XCIPs) occurs in the white blood cells of elderly females; this study was performed to determine whether this occurs in myeloid or lymphoid lineages. XCIPs were analysed in purified neutrophils and T cells from 80 females >75 years and the results were compared with 23 cord blood and 94 younger adult blood samples. The degree of XCIP skewing in cord blood and younger adult blood cells was similar, with 3–4% having >90% expression of one allele. Skewing was markedly increased in the neutrophils of elderly females, with 33% having >90% expression of one allele ($P < 0{\cdot}0001$). Extreme skewing was present in only 9% of the elderly T-cell samples and no evidence of T-cell clonality was found by PCR analysis of the TCRγ gene. The high level of acquired skewing of the XCIPs in myeloid cells of the elderly suggests that with time there is a change in stem cell usage with stochastic loss of some of the original stem cells. This has major implications for the use of XCIP analysis in the diagnosis of myeloid malignancies in the elderly and for gene therapy into haemopoietic stem cells.

Keywords: haemopoietic stem cells, stem cell pool size, clonal analysis, HUMARA.

All haemopoietic cells are ultimately derived from pluripotent stem cells through a series of differentiation pathways committing their progeny to the various different cell lineages (Till & McCulloch, 1980). The system has enormous reserve potential and haemopoiesis rarely fails, even in elderly patients who have been exposed to chemotherapeutic agents toxic to stem cells. Regulation of the stem cell pool is poorly understood but is of major importance to therapeutic strategies which involve the manipulation of such stem cells, including the introduction of exogenous genes.

In one model system, stem cells are considered to be capable of only a limited number of self-renewal divisions and once they have 'aged' their progeny are destined to enter the differentiated cell pool and be lost through committment (Rosendaal *et al*, 1979). Haemopoietic exhaustion is prevented by the presence of a vast excess of stem cells with relatively few being used at any one time (clonal succession) (Kay, 1965). In other models the stem cell pool is considered to be smaller but capable of continuous self-renewal. If the self-renewal occurs in a symmetrical manner, not only will the size of the stem cell pool remain constant but it will, throughout life, contain descendants of all the original stem cell pool members. There is, however, evidence that many of the commitment decisions in the haemopoietic system have a random or stochastic element (Till *et al*, 1964; Ogawa *et al*, 1983). Although the overall self-renewal rate at each division in the stem cell pool must be 0·5 to maintain the steady state, at the single cell level any one dividing stem cell could have a self-renewal rate of 1·0 (both daughter cells are stem cells), 0·5 (one daughter cell is a stem cell and one enters the differentiating cell pool) or 0 (both daughter cells undergo terminal differentiation). This latter model would predict that, with time, the stem cell pool would come to be made up of the descendants of fewer and fewer of the original stem cells but without a decline in the total number of stem cells.

The fate of individual stem cells has been studied in murine transplantation models using retroviral markers (Lemischka *et al*, 1986; Snodgrass & Keller, 1987). Such studies suggest that for some weeks or months after

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transplantation of stem cells into irradiated hosts there is evidence of considerable fluctuation in the active stem cells, consistent with the theory of clonal succession (Kay, 1965). With time this stabilizes and subsequently haemopoiesis appears to be maintained by just a few stem cells which presumably can self-renew as they are able to last for much of the life-time of the animal (Keller & Snodgrass, 1990). An alternative strategy for tracking the fate of stem cells relies on the process of Lyonization or X-chromosome inactivation which occurs in females early in embryogenesis to maintain the same dosage of X-linked genes as in the male (Lyon, 1961). Females are thus mosaics for the expression of X-linked genes and if the maternal and paternal gene products can be distinguished by a polymorphic marker, two populations of cells can be detected. Using glucose-6-phosphate dehydrogenase (G6PD) isoenzymes in cats, Abkowitz *et al* (1990, 1995) demonstrated significant changes in isoenzyme expression for several years after autologous transplantation and in most animals this eventually stabilized to expression of a single G6PD type.

However, transplantation involves dramatic perturbation of the haemopoietic environment and the outcome is influenced by the number of stem cells injected (Smith *et al*, 1991). Such studies do not necessarily reflect normal stable haemopoiesis. In control cats, for example, untreated by chemotherapy or transplantation, the isoenzyme patterns remained constant over the 6 years of the study, implying either that the active contributing stem cells were stable during this time, or that the number of active stem cells was sufficiently large that small changes would not be detectable (Abkowitz *et al*, 1990).

Similar longitudinal study of individuals has not been feasible in humans. For many years G6PD was the only suitable X-linked polymorphic marker and studies were limited by the low frequency of protein polymorphisms in Caucasians. However, with the introduction of techniques using DNA, X-chromosome inactivation patterns (XCIPs) can now be studied in most females using a number of X-linked loci. Maternal and paternal genes of informative females can be distinguished either by a restriction enzyme polymorphism, e.g. phosphoglycerate kinase (PGK) and hypoxanthine phosphoribosyl transferase (HPRT) (Vogelstein *et al*, 1987) or a variable number tandem repeat sequence, e.g. the DXS255 locus recognized by the probe M27β (Boyd & Fraser, 1990) and the human androgen receptor (HUMARA) gene (Allen *et al*, 1992). Active and inactive X-chromosomes have different methylation patterns which can then be distinguished using methylation-sensitive restriction enzymes, e.g. *Hha* I and *Hpa* II. Results using a PCR-based assay for HUMARA correlate well with those using Southern blotting for PGK, HPRT or M27β (Gale *et al*, 1996).

Using these techniques, we have previously studied XCIPs of leucocytes from haematologically normal females (Gale *et al*, 1991). Although they showed the expected Gaussian distribution about a mean of approximately 50% expression of the lower allele, consistent with a random process of X-chromosome inactivation, in many individuals we found a considerable skewing of the pattern, with >75% expression of one allele. We report here a study of myeloid and lymphoid cells from haematologically normal females from birth to old age which indicate that, with time, there are changes in XCIPs consistent with a loss of stem cells from the original haemopoietic stem cell pool.

MATERIALS AND METHODS

Samples. Peripheral blood from 197 females was divided into three age categories. 23 were cord bloods taken at the time of delivery; 94 were from young adults, age range 17–50 years, median 30 years; 80 were from elderly adults $\geqslant$ 75 years, range 75–96 years, median 81 years. All were haematologically normal, with normal full blood counts and white cell differentials for their age. All samples except 63 from young adults were separated into neutrophils and mononuclear cells using standard density gradient centrifugation (Lymphoprep from Nycomed, Oslo, Norway). T lymphocytes were purified from the mononuclear fraction using three different methods according to the number of cells available, either E-rosetting using aminoethyl-isothiouronium bromide hydrobromide treated sheep erythrocytes (Chanarin, 1989) (n=19), removal of monocytes using carbonyl iron ingestion (Rozenszajn *et al*, 1984) (n=38) or CD3-coated magnetic beads from DYNAL (New Ferry, U.K.) (n=77). Sample purity was assessed morphologically and was >75% (neutrophils range 75–100%, median 95%; lymphocytes range 75–100%, median 97%). High molecular weight DNA was prepared by proteinase K/detergent digestion with phenol/chloroform extraction and ethanol precipitation (Sambrook *et al*, 1989; Gustincich *et al*, 1991).

X-chromosome inactivation patterns. DNA was screened for heterozygosity of the PGK, HPRT and DXS255 loci and XCIPs analysed in informative individuals using Southern blotting as previously described (Gale *et al*, 1991, 1992; Gale & Linch, 1994). Analysis of the HUMARA gene was carried out using a radioactive PCR-based technique (Gale *et al*, 1996). Autoradiographic signals were quantified using a Hoefer scanning densitometer (Hoefer Scientific Instruments, San Francisco, Calif.) and results are reported as the mean percentage expression of the lower allele from two or more analyses.

T-cell receptor γ chain gene rearrangement. The γ chain of the T-cell receptor gene (TCRγ) was amplified using the method of Diss *et al* (1995). Reaction mixtures (50 μl) contained approximately 100 ng DNA, 1 × Taq buffer from Promega Ltd (Southampton) (10 mM Tris HCl, pH 9·0, 50 mM KCl, 0·1% Triton X-100), 1·5 mM $MgCl_2$, 200 μM dNTPs and 250 ng of each primer. Each sample was analysed using two sets of primers, Vγ I + Vγ III/IV with either Jγ 1/2 or JPγ 1/2. After denaturation at 95°C for 5 min, 0·5 unit Taq polymerase was added and 40 cycles performed, each 1 min at 95°C, 1 min 55°C, 1 min 72°C, with a final 5 min extension time at 72°C. Products were electrophoresed through 10% polyacrylamide, stained with ethidium bromide and visualized under UV light.

RESULTS

XCIPs in females of different ages

XCIPs were analysed in haematologically normal females

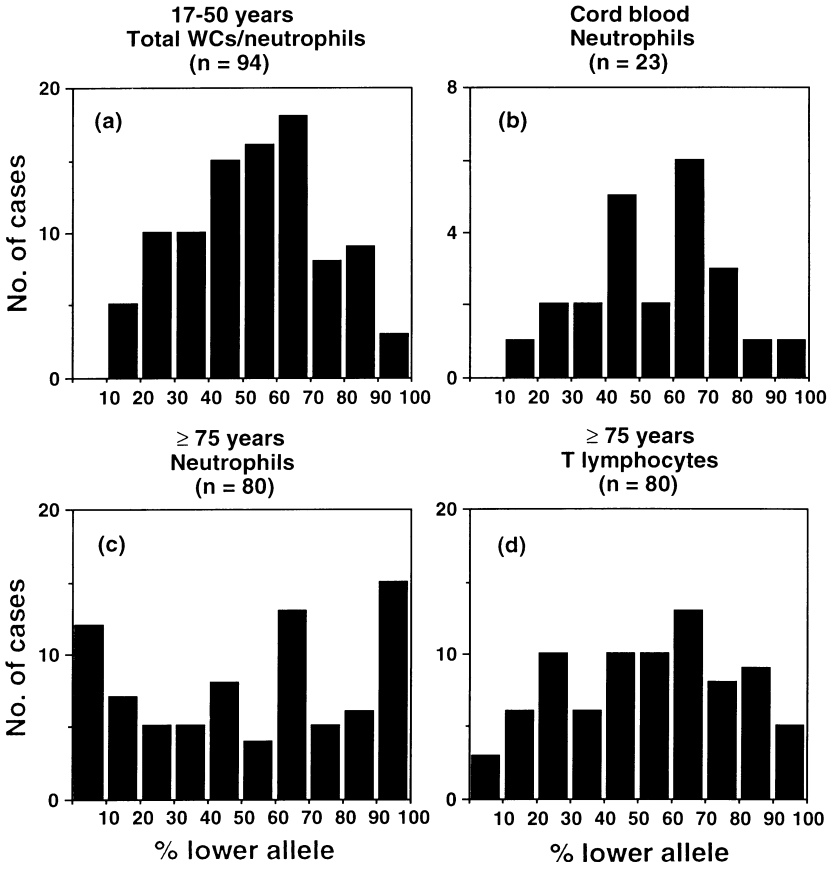

Fig 1. Distribution of X-chromosome inactivation patterns from peripheral blood of haematologically normal females in three different age groups.

from three different age groups, at birth (n=23), between 17 and 50 years of age (n=94) and ⩾75 years of age (n=80). Fig 1(a) shows the distribution of patterns obtained from leucocytes or neutrophils of 71 females aged between 17 and 50 years used as controls in our previous studies (Gale *et al*, 1993, 1994), and from neutrophils of a further 23 females in this age group analysed using the HUMARA assay. To ensure that the distribution of XCIPs observed in younger adults was a true reflection of the Lyonization pattern established early in life we analysed XCIPs of neutrophils purified from 23 female umbilical cord blood samples using the HUMARA assay. The degree of skewing was almost identical to that seen in the younger adults (Fig 1b). 26% of the cord blood neutrophil samples had >75% expression of one allele and 4% had >90% expression of one allele compared to 22% and 3% respectively in the younger adults (Table I).

This relatively high level of skewing suggests that the cell pool giving rise to haemopoietic stem cells is relatively small at the time of X-chromosome inactivation, with the skewing being a random but expected event. Combining data from the 117 cord blood and younger adult samples, it can be estimated that the pool size at the time of Lyonization is approximately six cells which accords with our own earlier estimate (Gale *et al*, 1991) and that of others (Buescher *et al*, 1985; Puck *et al*, 1992). In addition, comparison of XCIPs obtained from purified neutrophils and T cells of the cord blood samples and 31 of the younger adults showed ⩽20% difference in expression of the lower allele, which is our limit of technical variability (Gale *et al*, 1994) (Fig 2a). This is

Table I. Incidence of skewed XCIPs in haematologically normal females.

	> 75% expression of one allele	P*	> 90% expression of one allele	P*
17–50 years (total WBCs/neutrophils)	21/94 (22%)		3/94 (3%)	
⩾ 75 years (neutrophils)	45/80 (56%)	< 0·0001	26/80 (33%)	< 0·0001
⩾ 75 years (T cells)	32/80 (40%)	0·014	7/80 (9%)	0·19
Cord blood (neutrophils)	6/23 (26%)	0·7	1/23 (4%)	0·8

* The degree of significance as correlated to the young adult results.

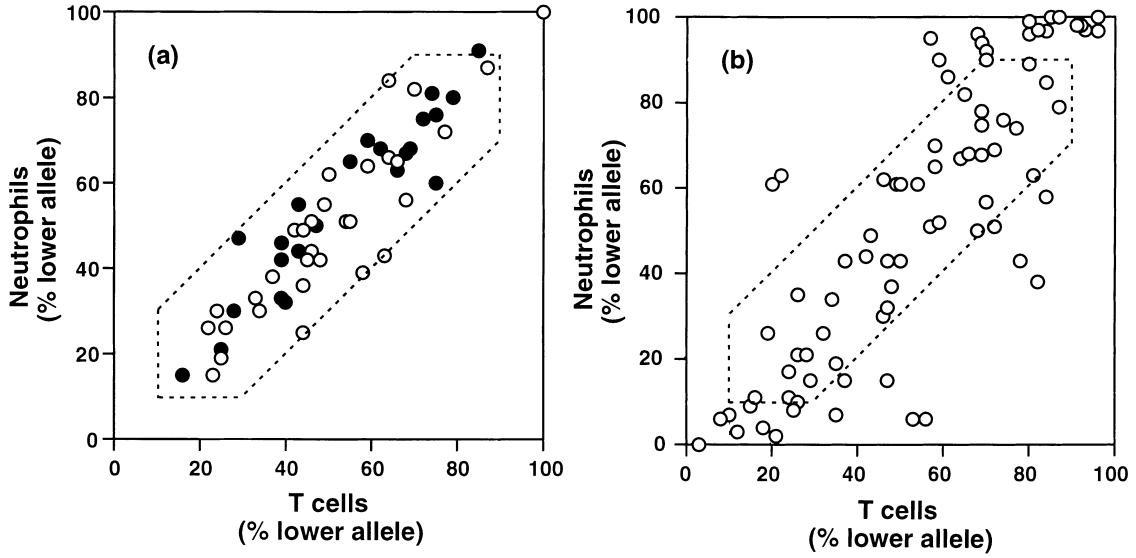

Fig 2. Comparison of X-chromosome inactivation patterns from neutrophils and T cells of haematologically normal females: (a) young adults (○), cord blood (●); (b) elderly adults. The dotted lines represent 20% difference in allele expression, the limit of our technical variation, and 90% expression of one allele.

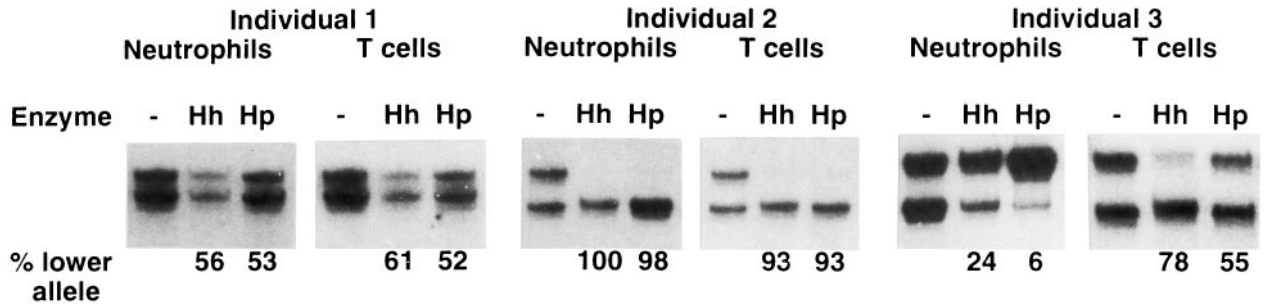

Fig 3. Representative HUMARA X-chromosome inactivation patterns from purified neutrophils and T cells of three females ⩾75 years of age. DNA is either undigested (-) or digested with *Hha* I (Hh) or *Hpa* II (Hp) before amplification.

consistent with the common stem cell origin of neutrophils and T cells (Gandini & Gartler, 1969; Keller *et al*, 1985).

XCIPs were studied in leucocytes from 80 females aged 75 years and over (range 75–96 years, median 81). 11 were analysed using Southern blotting in a study of XCIPs in different tissues (Gale *et al*, 1994) and 69 were new cases analysed using the HUMARA assay. These individuals were in hospital for a variety of non-haematological disorders and had normal peripheral blood counts and white cell differentials. For each individual, purified neutrophil and T-cell populations were studied as the development of T-cell oligoclonality in the elderly is well recognized (Posnett *et al*, 1994) and could be the cause of acquired XCIP skewing in a total leucocyte population. The results obtained were strikingly different from those in the younger individuals. Typical patterns obtained are shown in Fig 3. Neutrophil XCIPs in the elderly showed a marked increase in patterns at the extremes of the distribution (Figs 1c and 4). The incidence of XCIPs with >75% expression of one allele was 56% compared to 22% for the younger adult samples ($P <$ 0·0001, Table I). The difference was even greater if the incidence of imbalanced patterns with >90% expression of one allele was compared, 33% and 3% for the elderly and young adults respectively.

T-cell XCIPs in the elderly showed some broadening of the

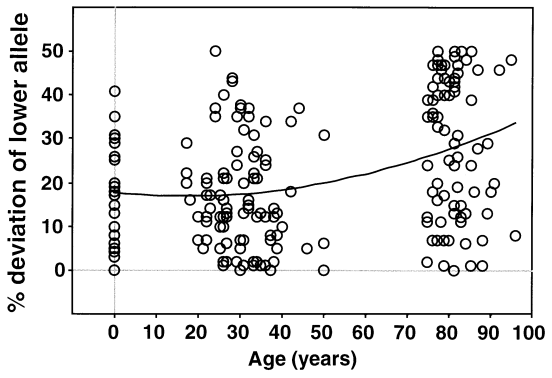

Fig 4. Increase in skewed X-chromosome inactivation patterns with age as shown by the percent deviation from 50% expression of the lower allele for each sample.

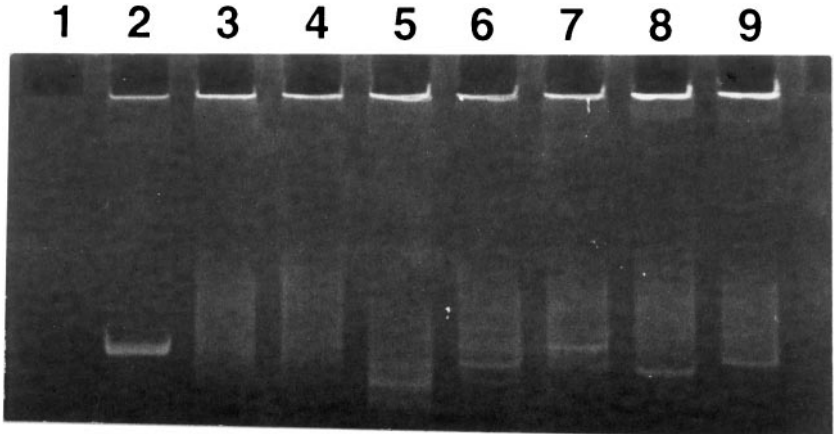

Fig 5. Representative TCRγ chain gene rearrangements of T cells from elderly adults. Lane 1: negative (water); lane 2: positive control; lanes 3–9: elderly females.

overall distribution but it was not as striking as in the neutrophils (Fig 1d). The difference in the incidence of XCIP skewing was significantly different from the younger female white cells for >75% expression of one allele (40% *v* 22%, $P = 0{\cdot}012$) but not for >90% expression of one allele (9% *v* 3%, $P = 0{\cdot}116$) (Table I). This difference between the degree of skewing in neutrophils and T cells in the elderly (56% and 40% respectively) presumably reflects the fact that neutrophils are short-lived whereas T cells are heterogenous, with some cells being produced recently from active stem cells but with many being long-lived and derived from earlier stem cells.

T-cell receptor γ chain gene rearrangements in the elderly

Although the loss of members of the original stem cell pool through terminal differentiation may frequently cause an acquired skewing of the neutrophil XCIP, in some individuals who have a constitutively skewed Lyonization pattern the loss of stem cells may be manifest as a shift towards a more balanced pattern. Such cases would usually be apparent from the disparity between their neutrophil and T-cell XCIPs and it is noteworthy that 10 elderly individuals (13%) had >20% difference between the percentage expression of the lower allele obtained for the two XCIPs without marked (>90%) skewing of the neutrophil population (Fig 2b). However, this can only be interpreted as evidence of loss of the original haemopoietic stem cell members if it is known that oligoclonal proliferation of the T cells has not occurred.

We therefore used a PCR-based technique to examine the TCRγ chain gene in DNA from the T cells of the elderly females (Diss *et al*, 1995). Although one single or several discrete bands could be detected in 23/57 individuals studied (40%), they were always minor clones against a polyclonal background (Fig 5), and the presence of detectable clones did not correlate with differences in T-cell and neutrophil XCIPs. There was no difference in the incidence of minor clones between individuals who had either greater or ⩽20% difference between the values for their T-cell and neutrophil XCIPs, 40% for both (6/15 and 17/42 respectively). Similarly, of the 34 individuals who had a polyclonal TCRγ pattern with both primer sets, nine had >20% difference in their XCIPs, including four with >40% difference in allele expression, i.e. percentage expression of the lower allele in their T cells and neutrophils was 22% and 63%, 82% and 38%, 53% and 6%, 56% and 6% respectively. In addition, of the five females with >75% expression of one allele in the T cells rather than neutrophils, a minor clone was detected in only one female. These results indicate that although oligoclonality of T cells is a frequent occurrence in the elderly, it cannot explain the differences observed in XCIPs.

Taken together, the presence of marked skewing (>90% expression of one allele) in XCIPs of neutrophils from 26 of the elderly individuals, and the significant discrepancy between neutrophil and T-cell XCIPs in a further 10, these results suggest that in approximately 45% of elderly individuals their haemopoiesis is derived from the descendants of only a few of the original stem cell pool members present at the time of Lyonization.

DISCUSSION

This study shows that there is considerable skewing of XCIPs in myeloid cells at birth, indicating that at the time of Lyonization there is only a small pool of approximately six cells destined to produce haemopoietic cells. As expected, T cells are similarly skewed, consistent with the presence of a common lympho-myeloid stem cell (Keller *et al*, 1985). This degree of constitutive skewing is not found in all tissues (Gale *et al*, 1994), which is in accord with data in the mouse embryo showing that X-chromosome inactivation occurs at different times in different tissues (Tan *et al*, 1993). The stem cell pool sizes for different tissues will therefore vary and for most tissues will be larger than that for the haemopoietic system. On a population basis the degree of skewing is shown to be constant for most of life (Fig 1a), but in the elderly there is an additional degree of acquired skewing of XCIPs in myeloid cells (Figs 1c and 4). The incidence of this acquired skewing is between 31% and 43% depending on whether the calculation is based on the degree of extreme skewing with >90% expression of one allele or also includes those cases where there is a significant difference in neutrophil and T-cell XCIPs. Fey *et al* (1994) and Busque *et al* (1996) have also shown an increased incidence in skewed XCIPs in elderly individuals, but both these studies used total leucocyte

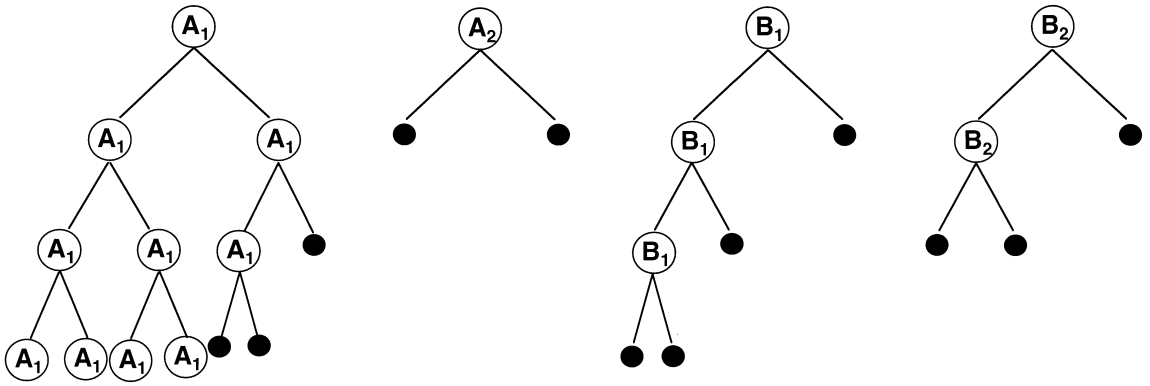

Fig 6. A model of stochastic clonal loss with age. The constitutive XCIP is determined by the relative pattern of expression of stem cells (A) or (B) at the time of Lyonization, e.g. with 50:50 expression of the two alleles as shown here. At the time of stem cell division, some cells will become committed to terminal differentiation (●). With time, although the absolute number of stem cells remains the same, some of the stem cells will be lost through terminal differentiation of both daughter cells. Haemopoiesis will therefore come to be derived from descendants of only a few of the original stem cell members and this will be reflected in the relative expression of the A and B alleles.

populations which did not take into account the possible acquired oligoclonality of the T cells (Posnett *et al*, 1994).

There are a number of possible explanations for this alteration in XCIPs. Firstly, the ability to distinguish between active and inactive X-chromosomes in these assays is dependent on their differential methylation patterns and these could change with age. However, although hypo- or hyper-methylation of specific loci has been demonstrated in some neoplastic disorders (Feinberg & Vogelstein, 1983; Goelz *et al*, 1985; Baylin *et al*, 1987), to account for the present observation the change would need to be selective for one X-chromosome and there is no evidence to support such a process in relation to ageing. Secondly, there may be a selective advantage of one X-chromosome. In female carriers of certain X-linked disorders such as Lesch-Nyhan syndrome and Wiskott-Aldrich syndrome, tissue-specific non-random X-chromosome inactivation has been demonstrated because the only cells which survive are those where the normal chromosome is active (Nyhan *et al*, 1970; Fearon *et al*, 1988). We cannot formally exclude this possibility.

Thirdly, the results could indicate stem cell depletion or exhaustion with age. Haemopoietic cellularity of the iliac crest bone marrow is reduced to approximately 30% of that in young adults by the age of 70 (Gilleece & Dexter, 1993) but idiopathic haemopoietic failure is exceedingly rare (Gordon-Smith, 1989). Furthermore, in the mouse the ability of elderly mouse marrow to support serial transplants is the same as that of young mice (Harrison & Astle, 1982). A fourth possibility is that the acquired skewing in the elderly is an early feature of myelodysplasia, a clonal 'pre-leukaemic' disorder of haemopoiesis, and is detectable before the typical blood changes of cytopenia and the morphological appearance of abnormal leucocytes. Myelodysplasia is more frequent in the elderly but the incidence in females in their eighth decade is still only 13 per 100 000 (Leukaemia Research Fund, 1990). Although a preleukaemic condition cannot be ruled out for a small number of individuals in our study, it is an unlikely explanation for our findings. Mutations in N-*ras* occur in approximately 20–30% of patients with myelodysplasia, in particular in codon 12 (Bar-Eli *et al*, 1989; Padua *et al*, 1988). We therefore amplified exon 1 of N-Ras by PCR and used a restriction enzyme digest specific for codon 12 mutations (Bashey *et al*, 1992) to screen neutrophils from 28 elderly females with a skewed XCIP. No mutations were detected (data not shown).

The most likely explanation for our findings is that the shift in XCIPs reflects a change in stem cell usage, with gradual loss of some of the original members of the stem cell pool present at the time of X-chromosome inactivation and expansion of others to keep the total number of stem cells relatively constant and thus maintain haemopoiesis. The constitutive XCIP of an individual is determined by the relative expression of X-linked genes which is laid down at the time of Lyonization, and in the model shown in Fig 6 this is represented by equal expression of A and B alleles, i.e. four stem cells with genotypes A_1, A_2, B_1 and B_2. At the time of stem cell division a decision is made between self-renewal to produce further stem cells or commitment to produce terminally differentiated cells, and this is regulated in such a way that the total number of stem cells is maintained. However, division may be either symmetric or asymmetric. Symmetric division would produce either two daughter stem cells, as shown in the model for cell A_1, or two terminally differentiated cells (cell A_2). Asymmetric division would produce one stem cell and one terminally differentiated cell (cells B_1 and B_2). With succeeding generations, if at least some of the stem cells underwent symmetric division, the stem cell pool would come to be descended from fewer of the original pool and this change would be reflected in the relative expression of the X-linked genes, for example, ultimately all four stem cells could be genotype A_1. The decision for stem cells of a particular genotype to self-renew or terminally differentiate would be random, consistent with a stochastic model of haemopoiesis. Under normal circumstances these shifts would be gradual and take many years to manifest. However, stress situations such as transplantation may accelerate the normal ageing process and lead to a more rapid acquisition of skewed patterns as demonstrated in the

studies in cats (Abkowitz *et al*, 1990, 1995, 1996). The finding of less acquired skewing in T cells of the elderly could be because the skewing process only occurs in a myeloid stem cell. Alternatively, it may be happening in the true lymphomyeloid stem cell pool and is masked in the T-cell population by the presence of a significant number of T cells formed earlier in life before acquired skewing occurred.

There are two important clinical implications from our findings. Firstly, XCIPs have been used as a tool in the diagnosis of myeloid malignancies by demonstrating clonality (Janssen *et al*, 1989; Tefferi *et al*, 1990; Tsukamoto *et al*, 1993). Clearly, this is inappropriate in the elderly patient where an imbalanced XCIP in neutrophils but not in T lymphocytes does not necessarily indicate the expansion of an abnormal clone. Secondly, the concept of stochastic clonal loss has implications for gene therapy using haemopoietic stem cells. If, as is currently the case, only a proportion of transplanted stem cells are successfully transduced (Brenner *et al*, 1993; Dunbar *et al*, 1995), in time, although in some individuals expression of the required gene will increase, in most it will decline as the transduced stem cells are lost through differentiation unless some selection/survival pressure can be applied.

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REFERENCES

Abkowitz, J.L., Catlin, S.N. & Guttorp, P. (1996) Evidence that hematopoiesis may be a stochastic process *in vivo*. *Nature Medicine*, **2**, 190–197.

Abkowitz, J.L., Linenberger, M.L., Newton, M.A., Shelton, G.H., Ott, R.L. & Guttorp, P. (1990) Evidence for the maintenance of hematopoiesis in a large animal by the sequential activation of stem-cell clones. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 9062–9066.

Abkowitz, J.L., Persik, M.T., Shelton, G.H., Ott, R.L., Kiklevich, V., Catlin, S.N. & Guttorp, P. (1995) Behaviour of hematopoietic stem cells in a large animal. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 2031–2035.

Allen, R.C., Zoghbi, H.Y., Moseley, A.B., Rosenblatt, H.M. & Belmont, J.W. (1992) Methylation of *Hpa* II and *Hha* I sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. *American Journal of Human Genetics*, **51**, 1229–1239.

Bar-Eli, M., Ahuja, H., Gonzalez-Cadavid, N., Foti, A. & Cline, M.J. (1989) Analysis of N-RAS exon-1 mutations in myelodysplastic syndromes by polymerase chain reaction and direct sequencing. *Blood*, **66**, 115–119.

Bashey, A., Gill, R., Levi, S., Farr, C.J., Clutterbuck, R., Millar, J.L., Pragnell, I.B. & Marshall, C.J. (1992) Mutational activation of the N-*ras* oncogene assessed in primary clonogenic culture of acute myeloid leukemia (AML): implications for the role of N-*ras* mutation in AML pathogenesis. *Blood*, **79**, 981–989.

Baylin, S.B., Fearon, E.R., Vogelstein, B., de Bustros, A., Sharkis, S.J., Burke, P.J., Staal, S.P. & Nelkin, B.D. (1987) Hypermethylation of the 5′ region of the calcitonin gene is a property of human lymphoid and acute myeloid malignancies. *Blood*, **70**, 412–417.

Boyd, Y. & Fraser, N.J. (1990) Methylation patterns at the hypervariable X chromosome locus DXS255 (M27β): correlation with X inactivation status. *Genomics*, **7**, 182–187.

Brenner, M., Rill, D., Holladay, M., Heslop, H., Moen, R., Buschle, M., Krance, R., Sanatana, V., French Anderson, W. & Ihle, J. (1993) Gene marking to determine whether autologous marrow infusion restores long-term haemopoiesis in cancer patients. *Lancet*, **342**, 1134–1137.

Buescher, E.S., Alling, D.W. & Gallin, J.I. (1985) Use of an X-linked human neutrophil marker to estimate timing of Lyonization and size of the dividing stem cell pool. *Journal of Clinical Investigation*, **76**, 1581–1584.

Busque, L., Mio, R., Mattioli, J., Brais, E., Blais, N., Lalonde, Y., Maragh, M. & Gilliland, D.G. (1996) Nonrandom X-inactivation patterns in normal females: Lyonization ratios vary with age. *Blood*, **88**, 59–65.

Chanarin, I. (1989) Immunological markers. *Laboratory Haematology: an Account of Laboratory Techniques*. Churchill Livingstone, Edinburgh.

Diss, T.C., Watts, M., Pan, L.X., Burke, M., Linch, D. & Issacson, P.G. (1995) The polymerase chain reaction in the demonstration of monoclonality in T cell lymphomas. *Journal of Clinical Pathology*, **48**, 1045–1050.

Dunbar, C.E., CottlerFox, M., O'Shaughnessy, J.A., Doren, S., Carter, C., Berenson, R., Brown, S., Moen, R.C., Greenblatt, J., Stewart, F.M., Leitman, S.F., Wilson, W.H., Cowan, K., Young, N.S. & Nienhuis, A.W. (1995) Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. *Blood*, **85**, 3048–3057.

Fearon, E.R., Kohn, D.B., Winkelstein, J.A., Vogelstein, B. & Blaese, R.M. (1988) Carrier detection in the Wiskott Aldrich syndrome. *Blood*, **72**, 1735–1739.

Feinberg, A.P. & Vogelstein, B. (1983) Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature*, **301**, 89–91.

Fey, M.F., Liechti-Gallati, S., von Rohr, A., Borisch, B., Theilkas, L., Schneider, V., Oestreicher, M., Nagel, S., Ziemiecki, A. & Tobler, A. (1994) Clonality and X-inactivation patterns in hematopoietic cell populations detected by the highly informative M27β DNA probe. *Blood*, **83**, 931–938.

Gale, R.E. & Linch, D.C. (1994) Investigation of methylation at *Hha* I sites using the hypervariable probe M27β allows improved clonal analysis in myeloid leukaemia and demonstrates differences in methylation between leukaemic and remission samples. *Leukemia*, **8**, 190–194.

Gale, R.E., Mein, C.A. & Linch, D.C. (1996) Quantification of X-chromosome inactivation patterns in haematological samples using the DNA PCR-based HUMARA assay. *Leukemia*, **10**, 362–367.

Gale, R.E., Wheadon, H., Boulos, P. & Linch, D.C. (1994) Tissue specificity of X-chromosome inactivation patterns. *Blood*, **83**, 2899–2905.

Gale, R.E., Wheadon, H., Goldstone, A.H., Burnett, A.K. & Linch, D.C. (1993) Frequency of clonal remission in acute myeloid leukaemia. *Lancet*, **341**, 138–142.

Gale, R.E., Wheadon, H. & Linch, D.C. (1991) X-chromosome inactivation patterns using HPRT and PGK polymorphisms in haematologically normal and post-chemotherapy females. *British Journal of Haematology*, **79**, 193–197.

Gale, R.E., Wheadon, H. & Linch, D.C. (1992) Assessment of X-chromosome inactivation patterns using the hypervariable probe M27β in normal hemopoietic and acute myeloid leukemic blasts. *Leukemia*, **6**, 649–655.

Gandini, E. & Gartler, S.M. (1969) Glucose-6-phosphate dehydrogenase mosaicism for studying the development of blood cell precursors. *Nature*, **224,** 599–600.

Gilleece, M.H. & Dexter, T.M. (1993) The biological aging of bone marrow. *Reviews in Clinical Gerontology*, **3,** 317–325.

Goelz, S.E., Vogelstein, B., Hamilton, S.R. & Feinberg, A.P. (1985) Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science*, **228,** 187–190.

Gordon-Smith, E.C. (1989) Aplastic anaemia; aetiology and clinical features. *Baillieres Clinical Haematology*, **2,** 1–18.

Gustincich, S., Manfioletti, G., Del Sal, G., Schneider, C. & Carninci, P. (1991) A fast method for high-quality genomic DNA extraction from whole human blood. *BioTechniques*, **11,** 298–301.

Harrison, D.E. & Astle, C.M. (1982) Loss of stem cell repopulating ability upon transplantation: effects of donor age, cell number, and transplantation procedure. *Journal of Experimental Medicine*, **156,** 1767–1779.

Janssen, J.W.G., Buschle, M., Layton, M., Drexler, H.G., Lyons, J., van der Berghe, H., Heimpel, H., Kubanek, B., Kleihauer, E., Mufti, G.J. & Bartram, C.R. (1989) Clonal analysis of myelodysplastic syndromes: evidence of multipotent stem cell origin. *Blood*, **73,** 248–254.

Kay, H.E.M. (1965) How many cell-generations? *Lancet*, **ii,** 418–419.

Keller, G., Paige, C., Gilboa, E. & Wagner, E.F. (1985) Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. *Nature*, **318,** 149–154.

Keller, G. & Snodgrass, R. (1990) Life span of multipotential hematopoietic stem cells in vivo. *Journal of Experimental Medicine*, **171,** 1407–1418.

Lemischka, I.R., Raulet, D.H. & Mulligan, R.C. (1986) Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell*, **45,** 917–927.

Leukaemia Research Fund (1990) Leukaemia and lymphoma: an atlas of distribution within areas of England and Wales, 1984–1988.

Lyon, M.F. (1961) Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature*, **190,** 372–373.

Nyhan, W.L., Bakay, B., Connor, J.D., Marks, J.F. & Keele, D.K. (1970) Hemizygous expression of glucose-6-phosphate dehydrogenase in erythrocytes of heterozygotes for the Lesch-Nyhan syndrome. *Proceedings of the National Academy of Sciences of the United States of America*, **65,** 214–218.

Ogawa, M., Porter, P.N. & Nakahata, T. (1983) Renewal and commitment to differentiation of hemopoietic stem cells (an interpretive review). *Blood*, **61,** 823–829.

Padua, R.A., Varter, G., Hughes, D., Gow, J., Farr, C., Oscier, D., McCormick, F. & Jacobs, A. (1988) RAS mutations in myelodysplasia detected by amplification, oligonucleotide hybridization, and transformation. *Leukemia*, **2,** 503–510.

Posnett, D.N., Sinha, R., Kabak, S. & Russo, C. (1994) Clonal populations of T cells in normal elderly humans: the T cell equivalent to 'benign monoclonal gammapathy'. *Journal of Experimental Medicine*, **179,** 609–618.

Puck, J.M., Stewart, C.C. & Nussbaum, R.L. (1992) Maximum-likelihood analysis of human T-cell X chromosome inactivation patterns: normal women versus carriers of X-linked severe combined immunodeficiency. *American Journal of Human Genetics*, **50,** 742–748.

Rosendaal, M., Hodgson, G.S. & Bradley, T.R. (1979) Organization of haemopoietic stem cells: the generation-age hypothesis. *Cell and Tissue Kinetics*, **12,** 17–29.

Rozenszajn, L.A., Radnay, J., Nussenblatt, R. & Sredni, B. (1984) Human lymphoid cells and their progenitors: isolation, identification, and colony growth. *Methods in Haematology*, **11,** 150–179.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory, New York.

Smith, L.G., Weissman, I.L. & Heimfeld, S. (1991) Clonal analysis of hematopoietic stem-cell differentiation in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, **88,** 2788–2792.

Snodgrass, R. & Keller, G. (1987) Clonal fluctuation within the hematopoietic system of mice reconstituted with retrovirus-infected stem cells. *The EMBO Journal*, **6,** 3955–3960.

Tan, S.-S., Williams, E.A. & Tam, P.P.L. (1993) X-chromosome inactivation occurs at different times in different tissues of the post-implantation mouse embryo. *Nature Genetics*, **3,** 170–174.

Tefferi, A., Thibodeau, S.N. & Solberg, L.A. (1990) Clonal studies in the myelodysplastic syndrome using X-linked restriction fragment length polymorphisms. *Blood*, **75,** 1770–1773.

Till, J.E. & McCulloch, E.A. (1980) Hemopoietic stem cell differentiation. *Biochimica et Biophysica Acta*, **605,** 431–459.

Till, J.E., McCulloch, E.A. & Siminovitch, L. (1964) A stochastic model of stem cell proliferation, based on the growth of spleen colony-forming cells. *Proceedings of the National Academy of Sciences of the United States of America*, **51,** 29–36.

Tsukamoto, N., Morita, K., Maehara, T., Okamoto, K., Karasawa, M., Omine, M. & Naruse, T. (1993) Clonality in myelodysplastic syndromes: demonstration of pluripotent stem cell origin using X-linked restriction fragment length polymorphisms. *British Journal of Haematology*, **83,** 589–594.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Preisinger, A.C., Willard, H.F., Michelson, A.M., Riggs, A.D. & Orkin, S.H. (1987) Clonal analysis using recombinant DNA probes from the X-chromosome. *Cancer Research*, **47,** 4806–4813.