Chromosomal mechanisms in murine radiation acute myeloid leukaemogenesis

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Chromosome 2 abnormalities, particularly interstitial deletions, characterize murine radiation-induced acute myeloid leukaemias (AMLs). Here, G-band analyses in CBA/H mice of early (1–6 month) post 3 Gy X-irradiation events in bone marrow cells in vivo and of karyotype evolution in one unusual AML are presented. The early event analysis showed that all irradiated animals carry chromosome 2 abnormalities, that chromosome 2 abnormalities are more frequent than expected and that interstitial deletions are more common in chromosome 2 than in the remainder of the genome. On presentation AML case N122 carried a t(2;11) terminal translocation which, with passaging, evolved into a del2(C3F3). Therefore two pathways in leukaemogenesis might exist, one deletion-driven, the other terminal translocation-driven involving interstitial genes and terminal genes respectively of chromosome 2. As all irradiated individuals carried chromosome 2 abnormalities, the formation of these aberrations does not determine individual leukaemogenic sensitivity as only 20–25% of animals would be expected to develop AML. Similar lines of argument suggest that chromosome 2 abnormalities are necessary but not sufficient for radiation leukaemogenesis in CBA/H nor are they rate limiting in leukaemogenesis.

Introduction
Animal models of human cancers are of value in aiding the understanding of mechanisms of tumour initiation and development. This is particularly the case for tumours induced by environmental agents such as ionising radiation. A large body of epidemiological evidence implicates ionising radiation as a causative agent for a broad range of human cancers (1); however, the identification of specific human cancers known to be caused by radiation is problematic. Acute myeloid leukaemia (AML*) is one radiation associated tumour for which mouse models are available. Inbred strains such as RFM, SJL/J, C3H/He and CBA develop AML at high frequency following exposure to various ionising radiations (2–9). The CBA/H strain is of particular value due to the very low spontaneous incidence of the tumour and the reproducible induction of AML in 20–25% of animals by a single acute exposure to 3 Gy X-irradiation (10,11).

Deletion of part of one mouse chromosome 2 homologue has been identified as a consistent feature of radiation-induced AML in many mouse strains (5,12–16). In CBA/H 96% of radiation-induced AMLs carry chromosome 2 abnormalities (14); furthermore, the chromosomal breakpoints involved in the abnormalities are non-randomly distributed along the length of chromosome 2 (14). While AMLs present with a mean latent period of ~18 months following 3 Gy acute X-irradiation (11), chromosome 2 abnormalities can be detected in haemopoietic cells at high frequency much earlier post-irradiation (13,14,17–19). This finding and the coincidence of chromosome 2 breakpoints observed in AMLs and early post-irradiation haemopoietic cells led to the suggestion that chromosome 2 loss/rearrangement is a very early and possibly initiating event in murine radiation induced AML (14). The detection of chromosome 2 aberrations in bone marrow cells presumed to be in the first metaphase following irradiation (i.e. at 24 h post-irradiation, Bouffler,S.D. and Meijne,E., unpublished observation) strengthens the case for chromosome 2 aberrations being initiating events.

While the majority of AMLs in CBA/H present with simple interstitial deletions in chromosome 2, a minority carry chromosome 2 translocations and insertions (14). The translocations are often non-reciprocal (i.e. terminal translocations). Terminal translocations, while uncommon, have been reported in a number of human haemopoietic malignancies and are, in some cases, characterized by clonal instability of the translocation event (20). In this report a study of early (1–6 month) post-irradiation chromosomal aberrations in bone marrow cells of CBA/H mice is presented together with an analysis of karyotype evolution in one AML.

Materials and methods
CBA/H strain mice were obtained from the MRC Radiobiology Unit, Chilton, UK and maintained under temperature and dark/light controlled conditions. Mice were allowed access to water and food ad libitum. All procedures conformed to the UK Animals (Scientific Procedures) Act, 1986. 10–20 week old male mice were whole body irradiated with 3 Gy 250 kVp X-rays. Direct metaphase preparations from femoral bone marrow cells were made by the method of Lee et al. (21) and G-banded according to Piper and Breckon (22). Analysis was performed using the automated karyotyping system described by Piper and Breckon (22). Full karyotypes were prepared for all cells analysed. Complete breakpoint assignments were made for all aberrations, these data are not presented but are available on application to the authors. Samples for karyotyping were obtained from mice at 1, 3 or 6 months following irradiation, and subsequently scored such that cytogenetic events could be allocated to individual mice.

The acute myeloid leukaemia N122 was obtained as a primary neoplasm following X-irradiation of male CBA/H mice and maintained by conventional in vivo passage through i.p. injection of leukaemic spleen cells into unirradiated female CBA/H recipient animals according to Breckon et al. (15). Recipients bearing the N122 AML were sacrificed when they showed marked enlargement of the spleen or were otherwise distressed; in the studies reported here ten such in vivo passages were performed.

Following sacrifice, the host tissues noted below were immediately sampled for the analysis of the AML karyotype. For the primary AML, karyotypes were obtained from direct cytogenetic preparation of bone marrow and spleen, also from short-term in vitro culture of concanavalin A (Con A)-stimulated spleen cells. At passages 2–6 direct cytogenetic analyses of spleen cells involved the scoring of relatively few cells simply to confirm the presence of characteristic marker chromosomes for this leukaemia (Figure 2A). At passage 7 an unusual infiltration of leukaemic cells into ascites fluid was noted; this

*Abbreviations: AML, acute myeloid leukaemia; ConA, concanavalin A; L, long marker.
promoted a more complete analysis of clonal cytogenetic contributions in short term cultures of this fluid. At passage 10, karyotypic analyses included metaphases from direct preparations of bone marrow and spleen and short-term Con A-stimulated cultures of ascites fluid.

In the data presented here detailed analysis of chromosomal abnormalities and clonal contributions were based on the scoring of 50-100 G-banded metaphases per tissue sample. Chromosome breakpoint allocation involved the analysis of at least 25 metaphases.

Results

Analysis of early post-irradiation events

A total of 651 bone marrow cell metaphases obtained from 20 animals receiving 3 Gy X-rays were analysed for stable aberrations by G-banding; of these, 448 cells were from 12 individuals sampled 1 month post irradiation, 89 from four individuals 3 months post irradiation and 14 from four individuals 6 months post irradiation. In total 295 stable aberrations were observed. This represents 466 chromosomal breakpoints throughout the genome scoring two breaks for reciprocal translocations and interstitial deletions and one break for terminal deletions and terminal translocations. Therefore the average level of stable damage in this sample of cells was 0.72 breakpoints per cell. For comparison, in a sample of 134 cells from control, un-irradiated mice, one terminal deletion was scored, giving a background frequency for stable aberrations of 0.007 breakpoints per cell. The distribution of the radiation-induced breakage events among the chromosomes is shown in Figure 1. Statistical comparison of the observed frequencies of events in chromosomes with the expectation on the basis of chromosome length (14) was made with \chi^2 tests. Over-representation of events was noted in chromosome 2 (P < 0.001) and chromosome 6 (P < 0.001). Breakpoints occurred significantly less frequently than expected in chromosomes X (P < 0.01), 18 and Y (both P < 0.05). The remaining chromosomes sustained and retained stable damage as expected from their size.

The spectrum of chromosome aberration types scored is presented in Table I. Considering the frequency of aberrations in all chromosomes, terminal translocations are most common, followed in descending order by reciprocal translocations, terminal deletions and interstitial deletions. In chromosome 2 the relative order, however, is different (Table I). Terminal translocations still predominate but the next most frequent aberration is the interstitial deletion class followed by reciprocal translocations and finally terminal deletions. This relatively excessive frequency of interstitial deletions is statistically significant (P < 0.01, \chi^2 test). The same trend of a relatively high frequency of interstitial deletions in chromosome 2 is apparent when data at 1, 3 or 6 months post-irradiation are considered separately (data not shown). Both chromosomes 1 and 3 also had 'preferred' aberration types (Table I), reciprocal translocations in the case of 1 (P < 0.05) and terminal translocations in the case of 3 (P < 0.01). Therefore, a relatively high frequency of interstitial deletion is not simply a feature of large chromosomes.

In the sample of 20 animals, all carried at least one chromosome 2 aberration; 11/20 animals carried chromosome 2 interstitial deletions and terminal translocations were scored in 12/20. Chromosome 2 reciprocal translocations were observed in 11/20 and terminal deletions in 3/20 animals. 9/11 of the interstitial deletions on chromosome 2 were similar in terms of breakpoint locations to those seen in AML cells (14). Evidence for early post-irradiation proliferative advantage of cells with this form of deletion was not especially strong. In one animal a possible clone carrying a del2(C3F3) was present in two of 23 cells scored, no other indications of clonal growth advantage associated with del2 were observed. The only other aberration possibly associated with growth advantage was a t(2;12)(B;ter) which was present in two of 19 cells scored.

Acute myeloid leukaemia N122

Primary N122. The principal clone identified in the primary N122 AML (Figure 2A) showed three translocations, t(6;10)(C2;D3); t(7;y)(D1;E); t(2;11)(H4;A4) and a deletion del4(A4C2). Trisomy of chromosomes 12, 15, 16 and 18 and monosomy13 were noted in some cells but no consistent numerical changes were noted. All karyotypically abnormal cells in the tissues examined carried the three translocations noted above implying that they were intrinsic to the N122 neoplasm. In a few metaphases the chromosome 4 deficiency was not apparent, perhaps suggesting that the deleted chromosome had been lost and the normal copy had been re-duplicated. Of particular note is the form of the t(2;11) and t(6;10) translocations. Both of these are most simply interpreted from breakpoint analysis as complete but non-reciprocal exchanges involving the telomeric regions of chromosomes 2 and 10 respectively. These events generate two distinctive long marker (L) chromosomes (derivatives of chromosomes 2 and 10) and were designated L1,(t(2;11)) and L2,(t(6;10)) respectively. Table II summarises the clonal contributions made by the L1 and L2 chromosome markers to the bone marrow (direct) and spleen (direct and short term in vitro culture) of the primary host animal.

Passage 7 N122. Karyotypic analysis of the leukaemic infiltration of ascites fluid by passage 7 N122 revealed a somewhat different and unusual karyotypic picture from that seen in the primary neoplasm. Instead of the consistent presence of both the L1 and L2 markers in these N122 metaphases, ~25% of karyotypically abnormal cells carried only the L2 marker, the L1 marker having been lost or rearranged such that it was unrecognisable.

Passage 10 N122. The clone containing the L1 and L2 markers, while remaining dominant, had been displaced to differing degrees in the tissues studied by the clone containing only the L2 marker (Table II).

The karyotype of the N122 clone carrying L2 alone is shown in Figure 2B and two points are notable. First, the L2 marker at passage 10 is identical to the t(6;10)(C2;D3) translocation that characterized the primary neoplasm; the
Table I. Spectrum of aberrations in bone marrow cells of 3 Gy X-irradiated CBA/H mice

<table>
<thead>
<tr>
<th>Aberration type</th>
<th>RT</th>
<th>TT</th>
<th>ID</th>
<th>TD</th>
<th>INV</th>
<th>INS</th>
<th>COMP/MAR</th>
<th>TOT</th>
</tr>
</thead>
<tbody>
<tr>
<td>All chromosomes</td>
<td>67(0.23)</td>
<td>103(0.35)</td>
<td>44(0.15)</td>
<td>45(0.15)</td>
<td>12(0.04)</td>
<td>9(0.03)</td>
<td>15(0.05)</td>
<td>295</td>
</tr>
<tr>
<td>All chromosomes except 2</td>
<td>14(0.26)</td>
<td>18(0.34)</td>
<td>15(0.28)</td>
<td>3(0.06)</td>
<td>2(0.04)</td>
<td>1(0.02)</td>
<td>0(0.00)</td>
<td>53</td>
</tr>
<tr>
<td>All chromosomes except 1</td>
<td>53(0.22)</td>
<td>85(0.35)</td>
<td>29(0.12)</td>
<td>42(0.17)</td>
<td>10(0.04)</td>
<td>8(0.03)</td>
<td>15(0.06)</td>
<td>242</td>
</tr>
<tr>
<td>All chromosomes except 3</td>
<td>12(0.38)</td>
<td>14(0.36)</td>
<td>4(0.11)</td>
<td>4(0.11)</td>
<td>0(0.00)</td>
<td>0(0.00)</td>
<td>2(0.05)</td>
<td>39</td>
</tr>
<tr>
<td>Chromosome 1</td>
<td>5(0.23)</td>
<td>9(0.33)</td>
<td>40(0.16)</td>
<td>41(0.16)</td>
<td>12(0.05)</td>
<td>9(0.04)</td>
<td>13(0.05)</td>
<td>256</td>
</tr>
<tr>
<td>Chromosome 2</td>
<td>65(0.23)</td>
<td>84(0.32)</td>
<td>42(0.16)</td>
<td>43(0.16)</td>
<td>11(0.04)</td>
<td>8(0.03)</td>
<td>14(0.05)</td>
<td>264</td>
</tr>
</tbody>
</table>

*RT, reciprocal translocation; TT, terminal translocation; ID, interstitial deletion; TD, terminal deletion; INV, inversion; INS, insertion; COMP/MAR, complex aberrations and unidentified markers; TOT, total. The main figure represents the scored frequency of the given aberration, the subscript in parentheses represents the relative frequency of the aberration type.

t(7;y)(D1;E) event was also present in all abnormal cells. Thus, this clone may be unambiguously judged to be a derivative of the primary AML clone. Second, the loss of the L1 marker was clearly not a non-disjunctional event. Instead the t(2;11)(H4;A4) L1 marker appears to have undergone a secondary intra-chromosomal rearrangement seemingly involving not only the original 11A4–2H4 telomeric translocation junction but also two interstitial sites (probably in the C and F region), within the chromosome 2 component of the original t(2;11) derivative chromosome. In this way the secondary derivative chromosome 2 appears as a simple interstitial deletion spanning the C–F region, i.e. typical of those chromosome 2 events seen in many other radiation-induced AMLs. Accompanying this complex change, in a high proportion of metaphases (~50%) the small derivative (terminally deleted) chromosome 11 had been lost and the normal homologue reduplicated.

**Discussion**

**Early post-irradiation events**

Chromosomal changes following a leukaemogenic dose of 3 Gy X-rays have been examined in CBA/H bone marrow cells. The system was minimally perturbed in that the strategy did not involve any transplantation of irradiated cells into hosts with ablated haemopoietic systems. Therefore the chromosomal changes and clonal developments observed should reflect as closely as possible the early cytogenetic status of a group of mice in which 20–25% (i.e. 4 or 5) would be anticipated to develop AML (10,11).

Over the post-irradiation time period considered, a clear excess of aberrations in chromosome 2 was noted (Figure 1). This is consistent with the earlier findings largely based on transplantation or in vitro assay (13,14,17,18). The pattern of chromosomal involvements reported here for CBA/H is somewhat different to that observed in C3H/He (18) and may represent inter-strain variation, although CBA and C3H strains are closely related (23). An excess of aberrations was also observed in chromosome 6 (Figure 1). We are unaware of chromosome 6 having been previously identified as being radiosensitive. In the absence of any clear chromosome 6 involvement in AMLs, the relevance of this finding to leukaemogenesis is at present unclear.

Considering the spectrum of aberration types (Table I), it can be seen that CBA/H mice are particularly prone to terminal translocation induction by radiation. Even if some of this
CBA/H chromosome 2 exhibits site-specific radiosensitivity, phenotypic effects at the stem cell compartment level leading to a proliferative advantage. The alternative hypothesis is that chromosome 2 events confer a strong proliferative advantage. It may be that the instability of the N122 t(2;11) translocation involves the intra-chromosomal interaction of telomeric arrays. The t(2;11) translocation junction appears to be telomeric, previous studies have shown the presence of inverted telomere-like repeat (TLR) sequences in 2C and 2F and implicated these in the genesis of the chromosome 2 deletions/incomplete rearrangements that characterize radiation induced AMLs (27). It may be that the instability of the N122 t(2;11) involves the intra-chromosomal interaction of telomeric arrays.

**Table II. Karyotypes in primary and passaged leukaemia N122**

<table>
<thead>
<tr>
<th>Karyotype (markers)*</th>
<th>Distribution of karyotypes (%)</th>
<th>Bone-marrow (direct)</th>
<th>Spleen (direct)</th>
<th>Spleen (in vitro)</th>
<th>Ascites (in vitro)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary AML</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 37 to 39 (L1 + L2)</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 39 to 41 (none)</td>
<td>0</td>
<td>35</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 40 to 42 (L1 + L2)</td>
<td>88</td>
<td>65</td>
<td>94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 80 to 84 (L1 + L2)</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Passage 10 AML</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 39 to 41 (none)</td>
<td>18</td>
<td>12</td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>n = 42 to 48 (L1 + L2)</td>
<td>62</td>
<td>80</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 42 to 48 (L2)</td>
<td>20</td>
<td>8</td>
<td>24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Normal diploid karyotype n = 40. L1 marker = t(1;2)(A4;H4); L2 marker = t(6;10)(C2;D2).

excess is due to misclassification caused by the relatively poor resolution of the G-banding technique, it remains that a high proportion of translocations involve a breakpoint region close to a telomere. This may be of importance given the gene-rich nature of chromosome ends (24, 25).

Chromosome 2 rearrangements are not only over-represented in 3 Gy irradiated mice but, amongst chromosome 2 events, the interstitial deletion (the most frequently found aberration in radiation induced AMLs) also is far more common than in the remainder of the genome. Again, some influence of scoring may account for this as well as genetic factors which render certain aberration types lethal to cells. Proliferative advantage of cells carrying interstitial chromosome 2 deletions as evidenced by clonal expansions was rare. The frequency of clones in CBA/H with aberrant chromosome 2 was less than that found by Hayata (17) in C3H/He or by Trakhtenbrot et al. (13) in SJL/J. These two studies involved either a transplantation strategy or in vitro culture of bone marrow cells which might have confounded estimates of clonal contributions. The effects of culturing or transplantation cannot, however, explain the difference between the data presented here and that from a similar study in CBA/Ca mice reported recently (19). The CBA/Ca study showed that following 2 Gy acute X-irradiation, cells with chromosome 2 abnormalities were observed in ~21% of mice and that the majority of these chromosome 2 aberrant cells showed clonal growth advantage. The present investigation in CBA/H failed to note any time dependent increase in the proportion of ch2 aberrant cells per mouse over 6 months, however all mice carried at least one ch2 aberrant cell. It is difficult to reconcile the differences between the two studies, particularly as the closely related mouse substrains, CBA/Ca and CBA/H were used.

Notwithstanding the differences between the studies, the CBA/H data presented here argue against the contention that the majority of early chromosome 2 events confer a strong in vivo proliferative advantage. The alternative hypothesis is that chromosome 2 exhibits site-specific radiosensitivity, gains strength from these observations but remains unproven. Certain chromosome 2 aberrant cells do nonetheless appear to have some in vivo proliferative advantage in CBA/H and it remains possible that chromosome 2 loss/rearrangement exerts phenotypic effects at the stem cell compartment level leading to preferential entry of chromosome 2 aberrant cells into the active, cycling population but not excessive clonal expansion. Such an effect might be obscured by the sampling of whole marrow cell populations. This may explain why chromosome 2 clonal contributions tend to be greater when scored after post-irradiation transplantation.

**Acute myeloid leukaemia N122**

At primary presentation, AML N122 although clearly of myeloid origin, was cytogenetically distinct from all other murine AMLs carrying chromosome 2 abnormalities on which we and others have reported previously. Although it is not possible to exclude sub-microscopic interstitial loss from the chromosome 2 component of the primary N122 t(2;11) translocation, this event appears to be cytogenetically complete. The other important characteristic of this event is that it appears to involve the interaction of the telomere of one copy of chromosome 2 with an interstitial region of chromosome 11. Non-reciprocal exchanges of this general form are represented in two other AMLs, N36 and N383 (14), but in these cases the exchange involved both chromosome 2 homologues and was incomplete, resulting in interstitial losses. Given that N122 is clearly malignant, it seems that gross losses from chromosome 2 were not essential for the initial development of this particular myeloid neoplasm. The translocation may have caused gene deregulation, in this respect the potential gene regulatory function of telomeres (reviewed, 26) might be relevant.

At passage 7 the primary t(2;11) translocation (L1 marker) was subject to a complex rearrangement resulting in the loss of all of the chromosome 11 component plus the C-F region of chromosome 2. The net outcome of this was an interstitially deleted chromosome 2 copy that was indistinguishable from that seen in many other primary AMLs; therefore, this secondary change left no clue as to the nature of the initial cytogenetic event that preceded it. The specific chromosomal sites involved in this secondary change in N122 are also important. The t(2;11) translocation junction appears to be telomeric, previous studies have shown the presence of inverted telomere-like repeat (TLR) sequences in 2C and 2F and implicated these in the genesis of the chromosome 2 deletions/incomplete rearrangements that characterize radiation induced AMLs (27). It may be that the instability of the N122 t(2;11) involves the intra-chromosomal interaction of telomeric arrays.

**Implications for mechanisms of leukaemogenesis**

The early event analysis demonstrates that chromosome 2 aberrations similar to those seen in AMLs (i.e. interstitial deletions) occur more frequently than expected from genome-wide data. The infrequent occurrence of these as clones in individuals suggests that it is not the case that all such aberrations have strong in vivo proliferative advantage. It may be concluded, therefore, that aberrations characteristic of AML form early and abundantly. The case of AML N122 indicates that some chromosome 2 deletions scored in AMLs may have formed as a secondary event to an initial chromosome 2 terminal translocation, therefore this class of aberration should be considered 'potentially leukaemic'. If 'AML-like' chromosome 2 deletions and chromosome 2 translocations are considered potentially leukaemogenic events, 16 of 20 animals could be considered to be 'initiated'. However, the expectation would be that only 4–5 of the 20 animals develop AML. This suggests that, at the cytogenetic level, chromosome 2 change, while necessary for AML development, it is not in itself sufficient. Similarly, since all animals carrying chromosome 2...
abnormalities are unlikely to develop AML, the formation of these aberrations so characteristic of AML should not be considered rate-limiting. Such rate limiting events might be represented by secondary gene or chromosomal mutations arising spontaneously within the persistent and, perhaps, relatively large pool of radiation-initiated myeloid target cells during the ~18 month mean post-irradiation latent period. The nature of such events remains obscure but published cytogenetic data provide no evidence for consistent structural chromosomal changes other than those involving chromosome 2. Overall, these observations are consistent with a leukaemogenic mechanism requiring interaction between radiation-induced chromosome 2 abnormalities and spontaneously arising sub-chromosomal mutations.

As all irradiated animals carried at least one chromosome 2 aberration, the formation of such aberrations does not determine which individual CBA/H mice will develop AML. The situation in CBA/Ca appears to be very different in that ~20% of irradiated individuals sustain stable chromosome 2 abnormalities and these were presumed to be the ~20% of animals destined to develop AML (19). Investigation of the spectra of early post-irradiation aberrations in other radiation AML sensitive and insensitive strains may help to clarify this apparent inconsistency.

To summarise, data presented here suggest the involvement of at least two chromosomal mechanisms for radiation myeloid leukaemogenesis, i.e. direct deletion of material from the interstitial region of chromosome 2 and chromosome 2 terminal translocation formation. The involvement of chromosomal regions encoding telomere sequence arrays may be a common theme for these mechanisms (27). Early post-irradiation (1–6 month) event analysis indicated that chromosome 2 aberrations occur early and in abundance in the majority of 3 Gy irradiated animals. Thus, while chromosome 2 alteration might be considered ‘initiating’ it does not determine which individuals will succumb to AML. Therefore, further events which appear not to be detectable at the cytogenetic level must be required before the development of overt AML.

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