Molecular cloning and characterization of a defective recombinant feline leukaemia virus associated with myeloid leukaemia

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The GM1 strain of feline leukaemia virus (FeLV) was isolated from a naturally occurring case of myeloid leukaemia and induces severe haematopoietic abnormalities, including myeloblastic leukaemia, on inoculation into cats. Molecular clones of FeLV-GM1 proviruses were obtained and studied by restriction enzyme mapping, blot hybridization and partial DNA sequence analysis. Two types of clone were isolated; the first was a replication-competent FeLV of subgroup A, resembling other low or minimally pathogenic FeLV-A isolates; the second was replication-defective with extensive deletions and mutations in \textit{gag} and \textit{pol}, although it has an intact \textit{env} gene of subgroup B phenotype. Large segments of the defective proviruses, from the 5' leader sequence upstream of the \textit{gag} gene to the 5' half of the \textit{env} gene, show structural hallmarks of endogenous FeLV-related proviruses. Infectious FeLV-GM1 viruses recovered after transfection were tested for their leukaemogenic potential in newborn cats. Early polyclonal myeloproliferative changes were observed in cats inoculated with FeLV-A/GM1 alone, although these were more pronounced in animals receiving the full FeLV-AB/GM1 complex reconstituted by cotransfection of the defective virus FeLV-B with its FeLV-A helper. Analysis of viruses in the bone marrow showed that replication of the subgroup B component is delayed and restricted to a proportion of cats. Most of the infected cats developed persistent abnormalities of haematopoiesis and one progressed to disseminated myeloid leukaemia. The defective recombinant FeLV-B/GM1 appears to play an indirect but important role in myeloid leukaemogenesis.

Introduction

Feline leukaemia virus (FeLV) is associated with a range of malignant diseases in its host, the domestic cat. The predominant form of neoplastic disease is T-cell lymphoma (lymphosarcoma), but a spectrum of other malignancies has been recorded, including myeloid leukaemia, erythroid leukaemia and multicentric fibrosarcoma (Hardy \textit{et al.}, 1976; Jarrett, 1984). Feline multicentric fibrosarcomas and T-lymphoid tumours have yielded numerous recombinant viruses with highly tissue-specific oncogenic potential (Besmer, 1983; Neil \textit{et al.}, 1987), but the viruses present in the rarer myeloid leukaemias have not been examined in detail until now.

The highly oncogenic feline leukaemia viruses isolated to date have proved to be recombinants in which FeLV replicative genes are replaced by a host cell-derived oncogene, but it is clear that there are other important determinants of FeLV pathogenesis, including envelope gene variation. By envelope phenotype, replication-competent FeLV isolates can be classified as subgroup A, B or C. Subgroup assay is based on interference to superinfection (Sarma & Log, 1971) and the phenomenon is assumed to follow the recognition and entry through distinct cell surface receptor structures by FeLV-A, -B and -C, and the blocking or down-regulation of the receptors by interaction with viral glycoprotein in productively infected cells. FeLV subgroup A viruses are associated with the entire spectrum of FeLV disease, since they are present in all isolates and appear to be necessary for efficient horizontal transmission (Jarrett & Russell, 1978; Jarrett \textit{et al.}, 1978). However, some isolates are only minimally pathogenic (Donahue \textit{et al.}, 1988). For subgroup B viruses, inoculation of these alone has
induced long latency thymic lymphosarcomas in isolated cases (O. Jarrett, personal communication) and such viruses are more commonly present in diseased rather than healthy viraemic cats (Jarrett et al., 1978). FeLV-B viruses appear to arise by recombination of FeLV-A with endogenous FeLV-related sequences (enFeLV) that harbour FeLV-B-like env genes (Stewart et al., 1986; Overbaugh et al., 1988a). In this and many other respects FeLV-B viruses provide a close parallel to mink-cell focus-forming (MCF) viruses of the mouse (Elder & Mullins, 1983; Neil et al., 1987). The importance of subgroup phenotype for disease potential is clearest for FeLV-C isolates, which invariably induce erythroid hypoplasia (Mackey et al., 1975; Onions et al., 1982; Riedel et al., 1986).

Feline leukaemia virus strain GM-1 was isolated from a naturally occurring case of myeloid leukaemia corresponding to stage M6 in the FAB classification (Bennet et al., 1982; Moore et al., 1974). The isolate produced a spectrum of myeloproliferative disease, including myeloblastic leukaemia, on passage in kittens. FeLV-GM1 contains a mixture of FeLV subgroups A and B, and preliminary evidence suggested that the subgroup B component might be the proximal leukaemogenic virus, since its replication increased significantly at onset of disease in chronically infected cats (Testa et al., 1988; Tzavaras et al., 1989).

This report concerns the molecular cloning and detailed characterization of proviruses from the FeLV-GM1 complex. The cloned viruses have been reconstituted by DNA transfection and tested for leukaemogenic potential in newborn cats. A spectrum of myeloid dysplasias and leukaemias has been reproduced.

Methods

Molecular cloning. DNA was isolated from FeLV-GM1-infected feline embryonic fibroblast (FEA) cells (Jarrett et al., 1973). Two bacteriophage libraries were used in cloning. Vector arms from Amersham (pWES EcoRI arms) and Northumbria Biologicals (pEMBL4 EcoRI arms) were ligated to EcoRI-digested and size-selected DNA (8 to 15 kb for pWES and 12 to 20 kb for pEMBL4) and packaged in vitro with standard extracts (Amersham). Several probes were employed in cloning the FeLV-GM1 proviruses. The defective subgroup B virus clones (pGMB-1 and pGMB-3) were derived by sequential high stringency screening with the exU3 probe [exogenous long terminal repeat (LTR) probe] (Mullins et al., 1984) and a subgroup B-specific env gene probe (B/S) (Stewart et al., 1986). The replication-competent FeLV clone was derived by sequential screening with exU3 and a subgroup A-derived probe, which has previously been shown to hybridize to FeLV-A and FeLV-C, but not to FeLV-B viruses (Stewart et al., 1986). EcoRI phage inserts obtained by screening the GM1 libraries were subcloned into the EcoRI site of the plasmid vector pUC18 (Norrander et al., 1983) for further analysis.

DNA transfection. Cells were transfected with proviral clones of FeLV using calcium phosphate precipitation, as adapted from the original method (Graham & van der Eb, 1973). Briefly, plasmid DNAs of FeLV clones (1 µg aliquots) were coprecipitated with carrier DNA (20 µg human placental DNA) in the presence of 140 mM-NaCl, 25 mM-HEPES pH 7-05, 75 mM-Na2HPO4, 125 mM-CaCl2 and the resultant precipitates were added to 1 x 106 FEA or AH927 (Rasheed & Gardner, 1980) cells which had been grown overnight in Ham’s F12 medium supplemented with 15% foetal calf serum. Medium was changed after 16 h and the cells were grown and subcultured for a further 3 weeks to allow virus spread. At this stage, transfected cell supernatants were tested for infectious virus by assay on C81 cells (Fischinger et al., 1974) and positive cultures were analysed for subgroup phenotype by the murine sarcoma virus (MSV) pseudotype assay (Russell & Jarrett, 1976). Further analyses of transfected cells were performed on DNA purified from the mass cultures at the same time.

Southern blot DNA analysis. High M, DNA was digested with restriction enzymes and separated by electrophoresis on agarose gels (0-8%). DNA fragments were transferred to GeneScreen membranes (DuPont) according to the manufacturer’s instructions. Hybridization was carried out according to previously published protocols (Neil et al., 1984). Specific probes were generated by nick translation of purified plasmid insert DNA to specific activities of 1 x 106 to 6 x 106 c.p.m./µg using commercially available kits (Amersham). In Southern blot analysis, a probe concentration of 5 ng/ml was used and hybridization was carried out at 42 °C for 16 h in a buffer containing 50% formamid, 0-1% SDS, 50 mM-sodium phosphate pH 7-5, 10% dextran sulphate. Blots were washed three times at 60 °C for 20 min in a buffer containing 0-5% SDS and either 0-1 x SSC or 2 x SSC for high or low stringency washes, respectively. After a final rinse at room temperature in 0-1 x SSC, filters were exposed to Kodak XAR-5 or XRP film at -70 °C.

DNA sequencing. DNA sequence analysis was carried out by the dideoxynucleotide chain termination procedure (Sanger et al., 1977). The pGMB-3 plasmid was further subcloned into M13 mp18 and mp19 (Norrander et al., 1983) and sequences were determined on both strands using T7 DNA polymerase in the commercially available Sequenase kit (U.S. Biochemical Corporation).

Bone marrow colony assays. Colony assays were carried out according to methods previously described for cat bone marrow. The granulocyte macrophage precursor cells (GM-CFC) were assayed in agar gel without the addition of exogenous colony-stimulating factor (CSF). Bone marrow cells were plated at either 1 x 105 cells/culture (1-25 x 105 cells/ml) or at 1 x 104 cells/culture (1-25 x 104 cells/ml). The lower concentration is one that we have previously shown to be limiting for the development of GM-CFC in the absence of exogenous CSF (Onions et al., 1982; Testa et al., 1983).

Infection of cats. Cats were infected within 48 h of birth with 5 x 104 p.f.u. of infectious FeLV-GM1 grown in FEA cells. One group of six cats received the helper virus alone, whereas the second group received the helper virus along with the defective subgroup B component. The two infected groups and the two age-matched control cats were housed separately. Peripheral blood samples and femoral bone marrow aspirates were obtained under anaesthesia.

These samples were used to determine the haematological and viraemic status of the cats, the numbers of bone marrow GM-CFC, and the structure and integration state of FeLV-GM1 proviruses in bone marrow DNA during the course of infection.

Results

Molecular cloning yields two classes of FeLV-GM1 provirus

The viruses present in the FeLV-GM1 complex were
length FeLV provirus (pGMA-3-2), which hybridized to both subgroups A and B. However, unlike most FeLV-A isolates, the FeLV-B/GM-1 virus could not be interference assays using MSV pseudotypes (Russell & Jarrett, 1976) showed that FeLV-GM1 contained viruses of both subgroups A and B. More detailed characterization of the FeLV-GM1 isolate was sought by molecular cloning of FeLV proviruses from cells infected with the virus complex. Two types of provirus were isolated. The first was a full-length FeLV provirus (pGMA-3-2), which hybridized to the FeLV-A-specific env probe virus and had a restriction map very similar to that of FeLV-A/Glasgow-1 (Stewart et al., 1986). The second type of structure was a truncated provirus which hybridized to an env gene probe specific for subgroup B FeLV. Two representatives of this proviral class (pGMG-1 and pGMB-3) were characterized in detail. The restriction maps of these clones are shown in Fig. 1 alongside a map of the prototype FeLV-A/Glasgow-1 clone. Restriction mapping indicated that the truncated proviruses are extensively deleted in gag and/or pol sequences.

The biological activities of the FeLV-GM1 clones were investigated after transfection into FEA cells (data not shown). These studies confirmed that the pGMA-3-2 clone had a full genetic complement and a subgroup A phenotype, whereas the truncated clones were replication-defective. The truncated clones could be rescued by cotransfection with the full-length helper virus clone and the resulting mixture had a subgroup AB phenotype. The subgroup B component routinely attained a copy number similar to that of the FeLV-A helper in cotransfection assays, despite its replication defect (Fig. 2). Digestion with KpnI/HindIII and blot hybridization with the exU3 probe readily differentiated the two components and showed that no secondary recombination had occurred to generate other FeLV-B species. This was of particular concern in view of the observation that DNA transfection of cultured feline fibroblastic cells can lead to the

\[ \text{Fig. 1. Molecular structure of clones isolated from FeLV-GM1. For comparison, the restriction map of a clone of FeLV-A/Glasgow-1 (pFGA-5) is shown at the top of the figure, below a gene map of the provirus. The structures of the replication-competent viral clone, pGMA-3-2, and two replication-defective clones, pGMB-1 and pGMB-3, are presented. Host cell sequences flanking integrated proviruses are not to scale. From sequence analysis the precise location of a 1.6 kb deletion in pGMA-3-2 and pGMB-3 structures. The stippled box encloses sequences that appear to originate from endogenous FeLV-related proviral sequences, as discussed in the text. Lines at the bottom of the figure show the extent of the sequence detailed in Fig. 3 and the SstI-XhoI fragment used as a hybridization probe in Fig. 5. Restriction enzyme site abbreviations are: B, BamHI; BgIII; E, EcoRI; H, HindIII; K, KpnI; P, PstI; S, SstI; X, XhoI.} \]
frequent generation of FeLV-B recombinants (Overbaugh et al., 1988a). In our studies, deliberate cotransfection with FeLV-B/GM1 forestalls the spread of any newly generated subgroup B virus, possibly by setting up an early blockade of host cell receptors. Digestion with KpnI and analysis with the B/S probe provided further confirmation that the FeLV-B/GM1 virus was stable during transfection and propagation.

Fig. 2. Replication of FeLV-A and defective FeLV-B viruses after cotransfection into FEA cells. Cells were transfected with proviral DNAs along with high M, carrier DNA (human placental DNA, 20 μg) and grown for 3 weeks. DNAs were digested with KpnI and hybridized with the B/S env probe (b) or digested with KpnI/HindIII and hybridized with the exU3 gene probe (a). The defective FeLV-B/GM1 virus generates a 3-7 kb KpnI fragment which can be detected by the B/S probe (designated B). Cross-reactive enFeLV sequences can be distinguished by mobility. The defective virus does not have a HindIII site in the env gene, so that KpnI/HindIII digestion leaves a 3-7 kb exU3-hybridizing fragment, easily distinguished from the 2-3 kb 3' fragment of the helper viruses FeLV-A/GM1 (pGMA-3-2) and FeLV-A/Glasgow-1 (pFGA-5) (labelled A). Cells were transfected with (lane 1) no viral DNA, (lane 2) pFGA, (lane 3) pFGA-5 and pGMB-1, (lane 4) pFGA-5 and pGMB-3, (lane 5) pGMA-3-2, (lane 6) pGMA-3-2 and pGMB-1 or (lane 7) pGMA-3-2 and pGMB-3. Molecular size markers (M) were end-labelled HindIII fragments of lambda DNA (kb).

FeLV-B/GM1 proviruses are extensively deleted in pol and carry multiple markers of recombination with endogenous FeLV sequences

Restriction mapping (Fig. 1) and hybridization analysis using fragments of pGMA-3-2, pGMB-1 and pGMB-3 as probes (data not shown) gave no indication that any of the clones contained a heterologous sequence insert (proto-oncogene) that might be responsible for the leukaemogenic properties of this virus isolate. Further information on structure and origin of the replication-defective FeLV-B/GM1 was therefore sought by DNA sequence analysis. The sequence of the 5' end of the FeLV-B/GM1 provirus in the pGMB-3 clone is shown in Fig. 3, where it is aligned with two exogenous FeLV and one enFeLV sequence.

The FeLV-GM1 LTR differs from those of the other exogenous FeLVs, Glasgow-1 and 1161E (Stewart et al., 1986; Donahue et al., 1988), by only one base insertion and two and eight point mutations, respectively. In the U3 domain, FeLV-B/GM1 diverges markedly from the sequence of several enFeLV-related proviral LTRs (Berry et al., 1988). As the exogenous FeLV U3 domain
Fig. 3. Sequence of 1247 bp of pGMB-3, extending from the 5' LTR (see Fig. 1). The GM1 sequence is shown in code. Matches in the other sequences are indicated by dots, whereas differences are shown in code or as gaps in the sequence. The sequences compared to pGMB-3 are from two exogenous FeLVs, FeLV-A/Glasgow-1 (pFGA-5: 5' sequence published here for the first time) and FeLV-A/1161E (Donahue et al., 1988), and one enFeLV sequence, CFE-6 (Berry et al., 1988). The boundaries of U3, R and U5 sequences are indicated, as are the inverted repeats (IR) marking the termini of the LTR, the tRNA primer-binding site (PBS), the consensus splice donor (sd) site for ene mRNA and the initiation codons for glycosylated and non-glycosylated forms of the FeLV gag precursor (gPr80 and Pr65pp).
enFeLV LTRs are active in directing the transcription of the FeLV gag gene sequences, even though some enFeLV reporter genes in transient expression assays (Berry et al., 1982, 1983). This raises the intriguing possibility that an enFeLV recombinant with exogenous FeLV may have acted as an intermediate in the transduction process.

Coding potential of the FeLV-B/GM1 gag gene

As shown in Fig. 4, the gag reading frame of FeLV-B/GM1 terminates within p12W of FeLV-A/1161E. In addition, six conservative and four non-conservative substitutions are seen. The glycosylated gag leader sequence is in frame with the main body of the gag gene but termination would occur within the p12W coding sequence. The Pr65g~ protein of FeLV-B/GM1 is also present in the Snyder-Theilen feline sarcoma virus, in which the residual gag gene sequences are fused to the fes oncogene (Hampe et al., 1982, 1983). This raises the intriguing possibility that an enFeLV recombinant with exogenous FeLV may have acted as an intermediate in the transduction process.

In the glycosylated gag leader sequence of FeLV-B/GM1 significant divergence from exogenous FeLVs is seen and here the sequence can be aligned much more closely to the CFE-6 enFeLV sequence. FeLV-B/GM1 and CFE-6 share a 9 bp insertion (bases 741 to 749) and multiple point mutations relative to exogenous FeLV sequences, as well as numerous individual base substitutions. In FeLV-B/GM1, the glycosylated gag leader sequence is in frame with the main body of the gag gene but termination would occur within the p12W coding sequence. The Pr65g~ protein of FeLV-B/GM1 is also prematurely terminated, 316 bases downstream of the initiation site. In addition, FeLV-B/GM1 shows a large deletion in pol relative to most exogenous FeLVs, which affects the reverse transcriptase coding sequence and spans base positions 2642 to 4273 in FeLV-A/1161E (sequence not shown).

The sequence of the env gene of FeLV-B/GM1 has not been determined, but hybridization to the B-specific env probe and its subgroup B phenotype provides evidence that at least the 5' portion of the gene is of endogenous origin (Stewart et al., 1986). The BamHI site proximal to the env gene is characteristic of enFeLV sequences (Soe et al., 1983, 1985). Also, the HindIII site in the FeLV-A env gene (see pFGA-5 and pGMA-3-2) is missing from the FeLV-B/GM1 clones, in common with other FeLV-B isolates and enFeLV.

Inspection of previously published FeLV sequences reveals that some of the enFeLV gag sequence hallmarks in FeLV-B/GM1 (e.g. the 9 bp insertion at 741 to 749) are also present in the Snyder-Theilen feline sarcoma virus, in which the residual gag gene sequences are fused to the fes oncogene (Hampe et al., 1982, 1983). This raises the intriguing possibility that an enFeLV recombinant with exogenous FeLV may have acted as an intermediate in the transduction process.

A possible endogenous FeLV progenitor of FeLV-B/GM1

In FeLV-B/GM1 the enFeLV-related sequences form a contiguous block of over 4 kb spanning the truncated gag-pol gene and at least the 5' end of env. We considered
that it might be possible to identify a progenitor of FeLV-B/GM1 by restriction mapping analysis of enFeLV in the feline genome. Fig. 5 shows a Southern blot hybridization analysis of genomic DNA from a variety of feline tissues and cell lines after digestion with SstI and XhoI, using the characteristic 3.5 kb SstI/XhoI fragment from FeLV-B/GM1 as a probe. Among the large number of fragments detected with this probe is a highly conserved but low or single copy number XhoI-SstI restriction fragment, which comigrates with the internal viral fragment. This fragment may represent a possible enFeLV progenitor of FeLV-B/GM1, assuming that significant alterations have not occurred during outgrowth in vivo.

The reconstituted FeLV-GM1 complex induces early expansion of GM-CFC and late myeloid leukaemia

To assess their leukaemogenic potential, FeLV-GM1 viruses isolated after DNA transfection were inoculated
into newborn cats. Cats were infected with the FeLV-A/GM1 virus alone or the full complex of FeLV-AB/GM1 and monitored at intervals after infection by bone marrow cell culture and hybridization analysis for FeLV sequences.

All the cats inoculated with the FeLV-AB/GM1 complex became viraemic and remained so throughout the course of the experiment. As early as 3 weeks post-infection (p.i.) an expansion in the GM-CFC compartment was found in all of the AB-infected cats compared to the control. In these infected cats, colonies were produced at a low inoculum of bone marrow cells (10^4 per culture) in the absence of exogenous CSF, indicating that an alteration in either response to, or production of, CSF had occurred (Table 1). An assay of 10^4 nucleated cells from normal bone marrow usually resulted in few (0 to 2) colonies, due to limiting endogenous CSF concentration at this cell density.

One of the cats in the AB-infected group (AB3) died at 7 weeks, after developing a wasting disease associated with enteritis and thymic atrophy. Immunosuppression caused by FeLV-AB/GM1 has been a feature of experiments with uncloned virus and is a concomitant of infection with other FeLV strains (Jarrett, 1984; Overbaugh et al., 1988b).

At 35 weeks cat A4 showed pronounced depression in GM-CFC numbers. This cat remained viraemic and at 34 weeks it died with a myeloproliferative condition in which there was a pronounced reduction of the erythroid, neutrophilic and granulocytic cell series; the eosinophil and basophil cell lineages were unaffected. There were numerous small foci of large blast cells, which had a high mitotic rate. More than 30% of the myeloid cells were blasts, but no infiltrations were seen in other organs. Cat A4 may therefore have died at an early stage in the evolution of AML.

The remaining viraemic cat (A3) stayed haematologically normal until the experiment was terminated, but a decrease in GM-CFC numbers was noted when the marrow was assayed at 38 weeks.

Early proliferation induced by FeLV-GM1 is polyclonal

Analysis of proviral structures and integration sites in infected cat bone marrow was undertaken to investigate the possibility that disease progression was associated with the development of further recombinant viruses, or that viral insertional mutagenesis might play a role in

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### Table 1. GM-CFC colony formation in cats infected with FeLV-AB/GM1*

<table>
<thead>
<tr>
<th>Cat</th>
<th>3 Weeks</th>
<th>35 Weeks</th>
<th>47 Weeks</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>GM-CFC 10^4 BM cells</td>
<td>GM-CFC 10^5 BM cells</td>
<td>GM-CFC 10^4 BM cells</td>
</tr>
<tr>
<td>Control</td>
<td>0 ± 1†</td>
<td>46 ± 6</td>
<td>0</td>
</tr>
<tr>
<td>AB1</td>
<td>10 ± 4</td>
<td>155 ± 18</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>AB2</td>
<td>12 ± 1</td>
<td>145 ± 14</td>
<td>D</td>
</tr>
<tr>
<td>AB3</td>
<td>11 ± 3</td>
<td>114 ± 6</td>
<td>Clusters only</td>
</tr>
<tr>
<td>AB4</td>
<td>14 ± 1</td>
<td>143 ± 30</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>AB5</td>
<td>18 ± 4</td>
<td>164 ± 23</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>AB6</td>
<td>16 ± 3</td>
<td>181 ± 19</td>
<td>Clusters only</td>
</tr>
<tr>
<td>AB7</td>
<td>15 ± 4</td>
<td>120 ± 1</td>
<td>22 ± 1.5</td>
</tr>
</tbody>
</table>

* The numbers of myeloid colony-forming units (GM-CFC) were determined by plating either 10^4 or 10^5 nucleated bone marrow (BM) cells in semi-solid medium without the addition of exogenous CSF, as described by Testa et al. (1983).

† Number of colonies was recorded ± the standard error.

‡ D, Dead.

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* The numbers of myeloid colony-forming units (GM-CFC) were determined by plating either 10^4 or 10^5 nucleated bone marrow (BM) cells in semi-solid medium without the addition of exogenous CSF, as described by Testa et al. (1983).
**Feline myeloid leukaemia virus**

Fig. 6. Replication of FeLV-GM1 viruses in bone marrow of infected cats. As in Fig. 2, digestion with *KpnI/HindIII* (lanes 2) and probing with exU3 is used to discriminate between helper FeLV-A/GM1 (2.3 kb) and the defective FeLV-B/GM1 (3.7 kb). Digestion with *HindIII* alone (lanes 1) releases junction fragments containing host and viral sequences of varying length according to the sequences present at the integration site. In (a) bone marrow from cats 4 weeks p.i. is shown and in (b) bone marrow from the same cats 38 weeks p.i.

<table>
<thead>
<tr>
<th>Cat</th>
<th>2 Weeks</th>
<th>36 Weeks</th>
<th>38 Weeks</th>
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<tbody>
<tr>
<td></td>
<td>GM-CFC</td>
<td>GM-CFC</td>
<td>GM-CFC</td>
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<tr>
<td></td>
<td>10⁴ BM cells</td>
<td>10⁴ BM cells</td>
<td>10⁵ BM cells</td>
</tr>
<tr>
<td>Control</td>
<td>1 ± 1† ND‡</td>
<td>0</td>
<td>60 ± 4</td>
</tr>
<tr>
<td>A1</td>
<td>2 ± 0 ND</td>
<td>12 ± 5</td>
<td>111 ± 11</td>
</tr>
<tr>
<td>A2</td>
<td>1 ± 1 ND</td>
<td>1 ± 1</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>A3</td>
<td>3 ± 1 ND</td>
<td>1 ± 1</td>
<td>42 ± 2</td>
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<tr>
<td>A4</td>
<td>20 ± 3 ND</td>
<td>0</td>
<td>1 ± 1</td>
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<tr>
<td>A5</td>
<td>8 ± 4 ND</td>
<td>2 ± 2</td>
<td>83 ± 12</td>
</tr>
<tr>
<td>A6</td>
<td>16 ± 6 ND</td>
<td>ND</td>
<td>ND</td>
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</tbody>
</table>

* The numbers of myeloid colony-forming units (GM-CFC) were determined by plating either 10⁴ or 10⁵ nucleated bone marrow (BM) cells in semi-solid medium without the addition of exogenous CSF, as described by Testa et al. (1983).

† Number of colonies was recorded ± the standard error.
‡ ND, Not done.
§ D, Died.

Table 2. *GM-CFC colony formation in cats infected with FeLV-A/GM1*  

This process. Southern blot analysis of DNA from the bone marrows of infected cats is shown in Fig. 6. Most FeLV isolates have at least one conserved internal *HindIII* site, in the *pol* gene, so that *HindIII* digestion of integrated proviruses and probing with LTR sequences reveals fragments that span the virus-host DNA junctions. The smear of hybridization in most of the *HindIII* digests indicates the presence of many different proviral junction fragments, none of which is represented in a significant fraction of the infected cells. These results suggested that the early GM-CFC expansion was polyclonal in nature. In case AB6, where disseminated myeloid leukaemia developed, a similar indistinct hybridization pattern was seen, but with a superimposed pattern of strong proviral junction fragments (Fig. 6b) showing the outgrowth of a dominant cell population. In the later marrow samples from the other cats in this experimental group (AB1, -3, -4, -5 and -7) there was some resolution to a simpler hybridization pattern, suggesting that these may also have been in the early stages of disease progression. The significance of a single strong junction fragment in the early sample from cat
AB5 is not clear, since it did not persist to the later sampling time.

**FeLV-B/GM1 replication in bone marrow is delayed and further restricted to a proportion of cats**

The association of the two proviral types with early disease development and later leukaemic progression was investigated by digestion of bone marrow DNA with KpnI/HindIII and Southern blot analysis. The restriction fragments detected with the exU3 probe differ for the defective (3-7 kb fragment) and helper (2 kb fragment) components. Sampling at 4 weeks p.i. showed that FeLV-A infection was well established even at this early stage, but the defective FeLV-B genome was undetectable. Later sampling of bone marrow (Fig. 6b; week 38) showed that the defective B genome was present at copy numbers equivalent to the FeLV-A genome in three out of five cases. The remaining two cats showed no detectable subgroup B genome. These results were confirmed using the B/S env probe. Cat A4, which remained viraemic until post-mortem, showed only the inoculum FeLV-A virus in bone marrow DNA (data not shown), thus no de novo recombinants were apparent. The remaining surviving cats in this experimental group, which had shown early, transient viraemia, showed no detectable FeLV infection in the post-mortem marrow samples.

**Discussion**

The GM1 strain of FeLV has been molecularly cloned and found to consist of two components which are both required to reproduce the full disease spectrum of the original isolate. The step-wise development of disease in FeLV-GM1-infected cats suggested that this will be a useful system in which to study host and viral determinants of myeloid leukaemogenesis.

The presence of endogenous FeLV sequence markers in FeLV-B/GM1 provides further evidence that subgroup B viruses are generated by recombination between exogenous FeLV-A and enFeLV-related sequences. However, unlike the replication-competent FeLV-B viruses characterized to date, FeLV-B/GM1 appears to have acquired its internally deleted gag-pol gene as well as the 5' end of its env gene from enFeLV.

A likely source of FeLV-B in vivo is from co-packaging of enFeLV transcripts during FeLV-A infection. Spread of genetically mixed viruses to uninfected cells could then lead to recombination to yield exogenous FeLV-B viruses. A similar hypothesis has been advanced to explain the frequent generation of FeLV-B viruses following transfection of feline fibroblast cell lines with FeLV-A proviral DNA (Overbaugh et al., 1988a). It is possible that defective viruses such as FeLV-B/GM1 are intermediates in the generation of replication-competent FeLV-B viruses; highly efficient propagation of FeLV-B/GM1 along with helper FeLV-A may have led to its expansion and survival as an unusually primitive form. Although a number of enFeLV proviruses have been molecularly cloned and characterized (Soe et al., 1983, 1985), none provides an obvious progenitor for FeLV-B/GM1. However, the hybridization analysis presented here reveals a potential low copy number progenitor, which is present in all of the cat DNAs that were tested. As yet we have no information as to whether this enFeLV is among the subset that is transcriptionally active in normal cat tissues (Soe et al., 1985).

The replication of FeLV-B/GM1 appears to be restricted at more than one level. Although the virus is readily propagated along with its helper *in vitro*, early bone marrow sampling of cats infected with the virus mixture showed only the helper FeLV-A genome at detectable levels. These results suggest that some early block exists to replication of the FeLV-B component. This might simply be due to a lack of cells with appropriate receptors for FeLV-B, but another more interesting possibility is that FeLV-A-induced expression of enFeLV env sequences blocks the receptors. Preliminary results suggest that this might be the case since FeLV-A/GM1-infected cat A4 showed high levels of enFeLV expression in bone marrow RNA, in contrast to the non-viraemic cats in the same experimental group (unpublished observations). A second level of restriction is suggested by the observed heterogeneity of progression to FeLV-B/GM1 replication in bone marrow later in infection. Such heterogeneity might be explained by a stochastic process which would have ultimately led to FeLV-B replication in all cases, but previous studies have provided evidence that relative resistance to FeLV-B exists. Attempts to infect large numbers of cats with biologically cloned and replication-competent FeLV-B isolates was successful in only 15 to 20% of cats (Jarrett et al., 1978). Although FeLV-B resistance might be due to a more efficient immune response, we should also consider the possibility that the outbred cat population is heterogeneous at a genetic locus analogous to the murine Rmcf locus, which confers resistance to MCF virus replication (Hartley et al., 1983).

Despite its strong accessory role, the action of FeLV-B/GM1 in leukaemogenesis is most probably indirect. The genome of the FeLV-B component could not be detected in bone marrow DNA of the cat with the most advanced disease and its replication in bone marrow was not strictly correlated with the stage of disease, as monitored by haematopoietic colony pattern. The molecularly cloned FeLV-GM1 isolate is immunosuppressive and it is possible that this property is
enhanced by the defective component. Defective viruses have recently been implicated as the active components of highly immunosuppressive variants of FeLV (Overbaugh et al., 1988b) and murine leukemia virus (MuLV) (Aziz et al., 1989; Hartley et al., 1989). It is intriguing to note that the immunosuppressive Duplan MuLV has as its only substantial open reading frame a variant gag gene of unknown origin (Aziz et al., 1989), which might suggest that the Duplan virus originated as a recombinant between exogenous MuLV and an defective endogenous virus.

The early polyclonal expansion of myeloid precursor cells associated with FeLV-GM1 is of particular interest, since it can be induced rapidly and reproducibly by viruses with no recognized oncogene. The mechanism is not yet clear, but it seems likely that early tissue-specific proliferation may be a prerequisite for later neoplastic disease (Testa et al., 1988). Our further studies will focus on the proliferative effect of molecularly cloned FeLV-GM1 viruses in vitro and in long-term bone marrow cultures.

We are grateful for the excellent technical assistance of A. Campbell, M. Golder and R. McFarlane. This work was supported by grants from the Leukaemia Research Fund and the Cancer Research Campaign. Ann McDougall received a studentship from the MRC AIDS Directed Programme.

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(Received 9 August 1989; Accepted 17 October 1989)