Retrovirus mediated transfer and expression of GM-CSF in haematopoietic cells

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Summary Two retroviral vectors were compared for their ability to express granulocyte-macrophage colony stimulating factor (GM-CSF) in a haematopoietic cell line, FDCP1, which is dependent on GM-CSF for survival. Both a MoMLV-based vector pVneoGM, and a MPSV-based vector, M3neoGM, were found to be capable of transmitting and expressing both GM-CSF and neomycin sequences in the myeloid FDCP1 cell line. Our results also demonstrate that pVneoGM is more efficient at generating GM-CSF independent colonies than M3neoGM. Analysis of cell lines derived after infection confirmed pVneoGM expressed higher levels of GM-CSF. Cell lines generated by infection with pVneoGM responded to levels of exogenous recombinant GM-CSF which did not stimulate growth of the parental cell line, suggesting autocrine stimulation may convey a proliferative advantage under sub-optimal growth conditions. Finally the parental vectors pVneo and M3neo were shown to be capable of expressing the neomycin gene in both murine haematopoietic progenitor and stem cells.

The clonal growth of haematopoietic cells in vitro relies on a group of proteins referred to as colony stimulating factors, or growth factors (Metcalf, 1984). Clonogenic assays have identified the target cell populations and physiological actions of many colony stimulating factors (Metcalf, 1984) and it seems that it is the ability of growth factors to maintain cell viability, trigger proliferation and direct differentiation patterns that make growth factors ideal candidates as the molecules that regulate haematopoesis in vivo (Metcalf, 1984; Sieff, 1987). Indeed, the perturbation of growth factor biochemistry could be a contributory factor in the multistep process of leukaemogenesis and may release a haematopoietic cell from normal growth restraints thus conveying a growth advantage to the cell (Eaves et al., 1986; Gordon et al., 1987; Heard et al., 1984; Gisselbrecht et al., 1987).

The haematopoietic growth factor GM-CSF can maintain the survival of relatively primitive multipotential cells (Metcalf et al., 1980, 1986) and is capable of directing the differentiation of progenitor cells down the granulocyte and macrophage lineages (Metcalf, 1980). Although the expression of GM-CSF is not detected in normal myeloid cells, GM-CSF mRNA and activity has been detected in the haematopoietic tissue from a number of patients with acute myeloid leukaemia (AML) (Young & Griffin, 1986; Young et al., 1987). In some of these samples, the autocrine production of growth factor permits cells to grow clonally in the absence of exogenous growth factor (factor independent). Both Laker et al. (1987) and Lang et al. (1985) have shown that introduction of a GM-CSF cDNA into the GM-CSF dependent myeloid cell line, FDCP1, which is dependent on either GM-CSF or IL-3 for its survival (Dexter et al., 1980) results in factor independent cell growth. The introduction of a GM-CSF cDNA into haematopoietic cells, therefore, allows a direct means of testing the potential of growth factors to contribute to the leukaemic process.

In this study, we have compared the ability of two retroviral vectors to express GM-CSF in the factor dependent FDCP1 cell line (Dexter et al., 1980). Retroviral vectors based on Moloney murine leukaemia virus (MoMLV) have been used to infect haematopoietic cells with some success (Lemischka et al., 1986; Dick et al., 1985). However, despite their ability to infect haematopoietic cells they are relatively inefficient in expressing long terminal repeat (LTR) driven sequences in these cells (Joyner et al., 1983; Williams et al., 1986; McVor et al., 1987). Myeloproliferative sarcoma virus, (MPSV) has a broader host range than MoMLV and is an ideal candidate from which to derive retroviral vectors (Stocking et al., 1986; 1985). It is important to understand the mechanism whereby different vectors express genes, as this will be reflected in the interpretation of the biological effects. We show that the retroviral vector based on MoMLV is more efficient at expressing GM-CSF in haematopoietic cells and that this vector is suitable for reintroduction of genes into haematopoietic stem cells.

Materials and methods

Cell culture and cell lines

Murine 3T3 fibroblasts, Ψ2 (Mann et al., 1983) and ΨAM (Cone & Mulligan, 1984) packaging cell lines and FDCP1 cells (Dexter et al., 1980) were grown in supplemented modified Eagles medium (SLM, Gibco 043-01136M), 10% fetal calf serum, 2 mM glutamine. The FDCP1 cell line was passaged in the presence of conditioned medium from the WEHI3B cell line as a source of IL-3 to a final concentration of 10% (Dexter et al., 1980). The FDCP1 cell line also proliferates in response to GM-CSF (Lang et al., 1985; Laker et al., 1987). In FDCP1 clonogenic assays, rGM-CSF stimulates maximal colony formation at between 219 to 875 pg ml⁻¹ (data not shown). Fibroblast cell lines containing the neomycin gene were selected and maintained in 800 µg ml⁻¹ of geneticin. The FDCP1 cell line was selected and maintained in 1 mg ml⁻¹ geneticin.

Generation of virus producing cell lines

Stable transfection of plasmids into the Ψ2 cell line was achieved by calcium phosphate co-precipitation (Graham & Van der Ebb, 1973). One µg of plasmid was mixed with 20 µg of sheared genomic carrier DNA and the DNA/CaPO4 precipitate was left on the cells overnight. The medium was then changed and the cells were incubated for a further 48-h expression period before selection in 800 µg ml⁻¹ geneticin. Genetin-resistant colonies were identified two weeks later and cell lines established from which virus stocks could be collected. Alternatively, the plasmids were transfected transiently in the presence of DEAE-dextran (Sompayrac & Danna, 1981) into the amphotropic virus packaging cell line

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VAM. Cells were exposed to 5–25 µg plasmid for 1 h. After a 48-h expression period, culture medium containing virus particles was removed, passed through a 0.45 µ filter and used immediately to infect the W2 packaging cell line. Genetin-resistant W2 virus-producing cell lines were then established.

**Virus infection (Magli et al., 1987; Mulligan, 1983)**

Virus-containing medium was harvested from virus-producing cell lines reseeded 24 h previously at 5 × 10^5 cells per 25 cm² flask, passed through a 0.45 µ filter and used immediately or stored at −20°C for up to 1 month. Fibroblasts to be infected were seeded out at 5 × 10^5 cells per 25 cm² flask one day before infection. The fibroblasts were exposed to an aliquot of virus (usually 50 µl) for 24 h in the presence of 6 µg ml⁻¹ polybrene after which the cells were washed and incubated for a further 48 h. Cells were then replated at 5 × 10^4 cells per 10 cm petri dish in medium containing genetin at a concentration of 800 µg ml⁻¹ with at least 3 dishes per point. Infected cells were incubated for 2 weeks after which colonies were counted. Virus titre was calculated taking into account the replating efficiency of the cells during the experiment. Bone marrow cells harvested as previously described (Pragnell et al., 1988) and FDCP1 cells were exposed to virus by co-cultivation with virus-producing cells seeded out 24 h previously at 5 × 10^5 cells per 25 cm² flask. Polybrene was added at 6 µg ml⁻¹ and WEHI-3B conditioned media at 10% (FDCP1 cells) or 2% (bone marrow cells). Co-cultivation took place for 24 h after which non-adherent cells were recovered. The non-adherent cells were washed twice in PBS to remove polybrene and growth factors before replating. For infections of primary bone marrow cells, after co-cultivation, only non-adherent cells were recovered. Nucleated cells were counted and the appropriate numbers of cells used for the clonogenic assays (see Materials and methods CFU-GM progenitor assay and CFU-A primitive progenitor assay).

**Clonogenic assays for haematopoietic cells**

**FDCP1 clonogenic assay** Infected FDCP-1 cells were replated into 0.3% agar or 0.9% methocellulose in supplemented alpha modified MEM containing 25% fetal calf serum at the desired cell density. WEHI-3B conditioned media was added to the dishes at 10% final volume and genetin at 1 mg ml⁻¹. The titration of recombinant GM-CSF in the FDCP1 clonogenic assay showed maximal colony formation between 219 to 875 pg ml⁻¹ with a replating efficiency of 16%. Colonies were counted after two weeks.

**CFU-GM progenitor assay and CFU-A primitive progenitor assay** (Pragnell et al., 1988) CFU-GM were assayed by culturing 1–5 × 10⁴ cells per 3 cm petri dish containing 0.3% agar on 0.9% methocellulose in supplemented alpha modified MEM containing 25% fetal bovine serum. Conditioned media from the AF1-97 or WEHI-3B cell lines were used as sources of colony stimulating factor. For detection of CFU-A, 10⁴ cells in 4 ml supplemented alpha modified MEM containing 25% fetal bovine serum in 0.3% agar or 0.9% methocellulose were incubated in the presence of 10% L929 conditioned media and AF1-97 (Pragnell et al., 1988) conditioned media in 6 cm petri dishes. Plates were incubated for 7 days for detection of CFU-GM and 11 days for detection of CFU-A colonies. Genetin was added at 1.5 mg ml⁻¹.

**Plasmids**

The plasmids used in this study are shown in Figure 1. M3neo (Laker et al., 1987) and M3neoGM were gifts from W. Ostertag. The GM-CSF cDNA insert (Gough et al., 1985) in M3neoGM was removed by digesting plasmid DNA with BamHI and purifying the insert. The BamHI GM-CSF fragment was then ligated into BamHI digested pVneo. The resultant construct was called pVneoGM. The plasmid pV200 neo (Episkopou et al., 1984) has been renamed in this study as pVneo, for ease of description.

**Molecular analysis of cell lines**

Genomic DNA and total cellular RNA were extracted by the method of Kreig et al. (1983). DNA samples were digested with restriction enzymes under the manufacturers recommended conditions and resolved by electrophoresis in 0.8% agarose gels containing Tris-acetate buffer. RNA samples (20 µg) were resolved by electrophoresis in 1.4% agarose gels containing formaldehyde and MOPS buffer (0.04 M morpholinepropane-sulphonic acid, 0.01 M sodium acetate, 1 mM EDTA, pH 7.5). Nucleic acids were transferred to Genescreen (Du Pont) membranes. Filters were probed with [α-32P] dCTP labelled, random primed 1 kb BglII/SmaI fragment from the neomycin phosphotransferase gene. Hybridisation was carried out in 50% (v/v) formamide, 5 × SSC, 5 × Denhardt's solution, 25 mM phosphate buffer, 0.1% w/v SDS and 100 µg ml⁻¹ of denatured salmon sperm DNA at 42°C overnight.
Results

Transfer of MolMLV and MPSV based retroviral vectors into fibroblast and haematopoietic cells

Figure 1 shows the retroviral constructs used in this study. The MolMLV based vector, pVneoGM, expresses the GM-CSF sequences directly from the viral LTR while expression of the neomycin gene is due to the thymidine kinase promoter (Episkopou et al., 1984). The retroviral vector M3neoGM relies on the viral LTR for expression of both the neomycin gene and GM-CSF sequences (Laker et al., 1987). GM-CSF will be either translated from a subgenomic viral transcript, as this vector retains viral splice donor and acceptor sequences, or as the second cistron of a bicistronic message.

The virus titres on NIH 3T3 fibroblasts ranged from 10⁴ to greater than 10⁵ genetic-resistant (G418) cfu ml⁻¹. Virus from all 4 vectors were capable of infecting the FDCP1 cell line, as measured by geneticin resistance (Table I). However, the MPSV vector M3neo was consistently 10-fold more efficient at infecting FDCP1 cells although there were no obvious differences in titre between the MolMLV or MPSV based vectors on NIH 3T3 fibroblasts.

Selection of virally infected FDCP1 cells for colony growth in the absence of exogenous growth factor (factor independence) demonstrates that the GM-CSF containing vectors express GM-CSF in the FDCP1 cells (Table I-G418, WEHI-CM). Factor independence is a direct consequence of expression of the GM-CSF sequences, as infection with the parental neo viruses did not generate any factor independent colonies after infection of an equivalent number of cells. From Table I it can be seen that pVneoGM virus generated 18-fold more factor independent colony than the M3neoGM virus. Since these vectors have equivalent titres on 3T3 and FDCP1 cells as determined by geneticin resistance, differences in frequency with which they convert FDCP1 cells to factor independence must be related to their two different modes of GM-CSF expression.

Growth factor release from Ψ2 cells harbouring GM-CSF vectors

Table II shows that conditioned medium from Ψ2 cells containing the retroviral GM-CSF constructs stimulated colony formation from bone marrow progenitors, whereas cells containing the vector alone did not. The colony stimulating activity released from the GM-CSF containing cells could be neutralised by antiserum to GM-CSF (DeLamarter et al., 1985), confirming the colony stimulating activity released is GM-CSF. Cells expressing pVneoGM released considerably more GM-CSF than cells expressing M3neoGM. A cell line harbouring M3neoGM released barely detectable levels of colony stimulating activity, despite the packaging cells ability to produce virus which could generate factor independent colony growth at a low frequency, after infection of FDCP1 cells (Table I).

Growth factor independence of FDCP1 cell lines generated by viral infection

Geneticin-resistant FDCP1 cell lines containing each of the 4 vectors were established in the presence of conditioned medium from the WEHI 3B cell line. These cell lines were tested for their ability to grow in a factor independent manner. Of the 6 cell lines tested which were infected with pVneoGM, 5 demonstrated clonal growth in the absence of exogenous growth factor. None of the FDCP1 cell lines which harboured the parental viral constructs could form colonies when replated in the absence of exogenous growth factor (Table III). Figure 2 shows that the correct transcripts corresponding to full length viral RNA and the shorter thymidine kinase promoter driven transcript are present in pVneo and pVneoGM infected FDCP1 cells, except in the single clone which did not replate into factor independent conditions (clone 12). This clone has no full length viral transcript which encodes the GM-CSF protein (Figures 1 and 2). DNA extracted from FDCP1 clones was digested with a restriction enzyme which cuts once within each viral LTR and analysed by Southern blot hybridisation (Figure 3). Cell lines infected with pVneoGM contain a 4.2 kb neo hybridising fragment corresponding to the expected size of the provirus, except for clone 12 which appears to contain a rearranged provirus (Figure 3).

Table III demonstrates that the replating of 4 cell lines containing M3neoGM in factor independent conditions reveals their inability to form colonies even when plated at 10⁴ cells ml⁻¹. Therefore, it is possible that, despite infection with a GM-CSF virus, the levels of expression of the GM-CSF sequences in many clones is not sufficient to allow factor independent colony growth.

The generally low and variable replating efficiency of the pVneoGM containing cell lines in factor independent conditions suggests that the level of autocrine secretion may not be high enough to efficiently sustain factor independent colony growth. To test whether cell lines generated after infection with pVneoGM were still responsive to exogenous growth factor and therefore not maximally stimulated by autocrine produced GM-CSF, a low level of recombinant GM-CSF (rGM-CSF) (De Lamarter et al., 1985) was added to the culture plates. Table IV shows that the two clones are still responsive to exogenously added rGM-CSF and their replating efficiencies are increased by its presence, while no such effect is observed on the parental FDCP1 cell line.

Infection of murine bone marrow progenitors and stem cells

To examine the feasibility of using these vectors to express GM-CSF in primary murine progenitor and stem cells, the parental neo viruses were used to infect bone marrow cells. The two in vitro assays used were the Cfu-GM progenitor assay (Pragnell et al., 1988; Metcalf, 1984) and the Cfu-A stem cell assay. Table V shows both M3neoGM and pVneo viruses could confer resistance to geneticin in murine progenitor and stem cell assays; 20–75% of clonogenic cells appear to be infected by these viruses as assayed by geneticin resistance. Examples of geneticin resistant Cfu-A colonies are shown in Figure 4. Therefore, these vectors would be suitable candidates for the introduction of GM-CSF in primary murine bone marrow cells.
Table II  Growth factor release from \( \Psi_2 \) cells

| Vector      | Clone | Anti-serum against GM-CSF | Colonies/10^3 cells plated |
|-------------|-------|---------------------------|----------------------------|
| pVneo       | 2     | -                         | 0                          |
| pVneoGM     | 3     | -                         | 52                         |
| pVneoGM     | 7     | +                         | 0                          |
| M3neo       | 4     | -                         | 0                          |
| M3neoGM     | 6     | -                         | 2                          |
| recombinant GM-CSF | | + | 0 |

\( 5 \times 10^2 \) virus producer cells were seeded into 25 cm² flasks in 5 ml medium. After 3 days the medium was removed and filtered. Three-day conditioned medium (CM) from \( \Psi_2 \) virus producer clones was assayed for growth factor activity using the CFU-GM progenitor assay. 100 µl of the test CM was added to 3 cm petri dishes and overlayed with 1 ml of 0.3% soft agar containing \( 7.5 \times 10^6 \) nucleated bone marrow cells. Antiserum and test CM were incubated for 30 min at 37°C before addition to the culture dishes. Colonies of greater than 30 cells were counted after 7 days. Each CM was tested twice with at least two dishes each experiment and the results averaged. Recombinant GM-CSF (De Lamarter et al., 1985) at 875 pg ml\(^{-1}\) was used as a control.

Table III  Colony formation in semi-solid medium of G418 resistant FDCP1 cell lines generated by infection with retroviral vectors

| Virus       | Clone | Relative Platinga Efficiency (%) | Colony Formation in WEHI-CM | Relative Platinga Efficiency (%) | Colony Formation in WEHI-CM |
|-------------|-------|---------------------------------|-----------------------------|---------------------------------|-----------------------------|
| pVneo       | 1     | 124 (25)                        | 0                           | M3neo                           |
|             | 2     | 50 (10)                         | 0                           | 13                              |
|             | 3     | 114 (5)                         | 0                           | 15b TNTC (44)                   |
|             | 4     | 98 (15)                         | 0                           | 16a TNTC (7)                    |
|             | 5     | 200 (4)                         | 0                           | 60                              |
|             | 6     | 100 (2)                         | 0                           | 17                              |
| pVneoGM     | 7     | 77 (33)                         | 60                          | M3neoGM                         | 13 | 180 (5) |
|             | 8     | 20 (15)                         | 7.5                         | 14                              |
|             | 9     | 72 (17)                         | 15                          | 15b TNTC (41)                   |
|             | 10    | 107 (7)                         | 10                          | 18                              |
|             | 11    | 58 (22)                         | 4                           | 19b TNTC (6)                    |
|             | 12    | 70 (7)                          | 0                           | 20b TNTC (7)                    |

Individual genetic-resistance colonies generated after infection with the vectors were picked and maintained under selective conditions. *Relative plating efficiency is the ratio of colony formation under selective conditions to colony formation under non-selective conditions as in Table II. Replating efficiency of the cell lines under non-selective conditions are shown in brackets as a percent. The cells were plated out at \( 10^4 \) cells ml\(^{-1}\) except for\(^{a}\) which were plated out at \( 10^5 \) cells ml\(^{-1}\). Three 3 cm plates containing 1 ml of semi-solid medium were used per point. TNTC colony numbers were too numerous to count. At least 50 colonies per control plate were counted.

Table IV  Response of pVneoGM infected FDCP-1 cells to exogenous GM-CSF

| Clone       | Cell densitya (ml) | Relative plating efficiency (%) | Relative plating efficiency (%) |
|-------------|---------------------|---------------------------------|---------------------------------|
| Parental FDCP-1 | 5 \times 10^4 | 20                              | 0                              |
| 21          | 5 \times 10^3        | 4.5                             | 130                             |
| 22          | 5 \times 10^3        | 0.5                             | 158                             |

Two clones harbouring the pVneoGM virus were plated out under non-selective and selective conditions as in Tables II and III. rGM-CSF = recombinant GM-CSF. RGM-CSF (De Lamarter et al., 1985) was added to the dishes to a final concentration of 0.875 pg ml\(^{-1}\) (0.1% rGM-CSF). The amount of rGM-CSF added was determined by titration of rGM-CSF in a FDCP1 clonogenic assay (data not shown). Maximal FDCP1 colony formation was between 219 to 875 pg ml\(^{-1}\) rGM-CSF. *Triplicate 3 cm petri dishes containing 1 ml of semi-solid medium and the appropriate cell density were used for each point.
Discussion

Retroviral vectors have been shown to infect primitive haematopoietic cells and integrate into the host genome with a high efficacy (Dick et al., 1985; Lemischka et al., 1986), yet levels of viral expression are disappointingly low (Joyner et al., 1983; Williams et al., 1986; Mcclvor et al., 1987). It is convenient to assess the efficiency of retroviral mediated gene expression in haematopoietic cell lines. Two previous studies (Lang et al., 1985; Laker et al., 1987) used the FDCP1 cell line as a recipient for GM-CSF containing retroviral vectors. The study by Lang et al. (1985) relied on the splice type vector pZIPneoSV (X) 1, whereas Laker et al. (1987) used mainly the MPSV based splice type vector M3neo. GM-CSF independent cell lines generated by these two studies differed in that, in contrast to cell lines studied by Laker et al. (1987), those studied by Lang et al. (1985) did not require the release of autocrine produced GM-CSF in order to stimulate cell proliferation. It is possible that these results do not contradict one another but differ owing to the vector used to express the GM-CSF sequence, and thus the levels of GM-CSF protein produced.

Table V  Infection of bone marrow progenitor and stem cells detected using in vitro colony forming assays

| Virus    | *G418 | Colonies/10^3 cells | % infection |
|----------|-------|---------------------|-------------|
| pVneo    | -     | 61                  | 74          |
|          | +     | 45                  |             |
| M3neo    | -     | 20                  | 40          |
|          | +     | 8                   |             |
| M3neo    | -     | 82                  | 28          |
|          | +     | 23                  |             |
| Control  | -     | 48                  | 0           |
|          | +     | 0                   |             |
| Control  | -     | 96                  | 0           |
|          | +     | 0                   |             |

A. CFU-GM progenitor cells

B. CFU-A stem cells

Figure 2  Northern blot analysis of RNA extracted from FDCP1 cell lines. Lane 1, parental FDCP1 cell line. Lanes 2–4, cell lines infected with pVneo. Lanes 5–8, cell lines infected with pVneoGM. Lane 7 contains RNA from clone 12. The mobilities of the 28S and 18S ribosomal mRNAs are marked.

Figure 3  DNA blot hybridisation analysis of proviral sequences. All DNAs digested with SstI, which cuts once within each LTR. Lane 1, parental FDCP1 DNA. Lanes 2–4, DNA from cell lines harbouring pVneoGM. Lane 7 is DNA extracted from clone 12. Sizes are in kilobases.
In order to compare the two vectors pVneoGM and M3neoGM for their ability to express GM-CSF in haematopoietic cells, the immature GM-CSF dependent myeloid cell line FDCP1 (Dexter et al., 1980) was chosen as a recipient for the vectors. The growth characteristics of FDCP1 cells immediately after viral infection reveal that pVneoGM converts a higher frequency of cells to factor independence than M3neoGM (Table I). The analysis of cell lines derived from infection uncovers a complexity hitherto unobserved. FDCP1 cell lines which contain the pVneoGM virus and which were initially selected for geneticin resistance demonstrate a variable and generally low replating efficiency under conditions which select for factor independent growth (Table III). This may relate to clonal variation in autocrine directed GM-CSF stimulation, which may be sub-optimal for efficient colony formation. Two FDCP1 clones which harboured pVneoGM increased their replating efficiency in response to low levels of exogenous GM-CSF (Table IV). This suggests autocrine stimulation is relatively inefficient yet sufficient to convey a proliferative advantage over the parental FDCP1 cell line under sub-optimal growth conditions.

Geneticin-resistant FDCP1 cell lines derived after infection with M3neoGM did not replate into growth factor independent conditions (Table III). These data suggest that M3neoGM expresses GM-CSF at a lower level than pVneoGM and at a level incompatible with factor independent clonal growth (Table III). The frequency with which M3neoGM converts the factor dependent FDCP1 cell line to factor independence is 50-fold lower than its ability to convey geneticin resistance (Table I). Therefore, in infections with M3neoGM virus many geneticin-resistant clones may have to be screened in order to identify those releasing sufficient GM-CSF to form colonies in the absence of exogenous growth factor.

The two parental viruses, pVneo and M3neo were assessed for their ability to infect and express the neomycin gene in primary murine bone marrow. Both vectors were able to generate geneticin-resistant CFU-GM progenitor colonies and CFU-A in vitro stem cell colonies (Table V). This is the first instance of the novel CFU-A assay (Pregnell et al., 1988) being used as a test system for retroviral expression experiments. The retroviral expression of genes in short term in vitro assays may give some indication as to the multiple steps involved in leukaemogenesis before, or as an alternative to, more complex in vivo experiments. Therefore, the use of short term in vitro assays to assess the efficiency of new vectors in terms of gene expression in haematopoietic cells is important. This study and others (Lang et al., 1985; Laker et al., 1987) have gone some way to examine the efficiency of retroviral vector mediated gene expression and address whether autocrine stimulation results in a proliferative advantage over non-autocrine stimulated cells.

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Fig. 4 Geneticin-resistant CFU-A colonies. Bone marrow cells were infected with M3neo as described in Table V. The diameter of the dishes is 6 cm. A, mock infection without geneticin. B, mock infection plus 1.5 mg ml⁻¹ geneticin included in the semi solid-medium. C, Infection with M3neo without geneticin. D, Infection with M3neo with 1.5 mg ml⁻¹ Geneticin included in the semi-solid medium.

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