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RESEARCH ARTICLE

Development of a suspension packaging cell line for production of high titre, serum-resistant murine leukemia virus vectors

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To date, only adherent cell lines have been used for the generation of packaging cells for the production of type C retrovirus vectors. The large-scale production of high titre retrovirus vectors could benefit from the development of packaging cells growing in suspension. Here, we describe the ability of two different lymphoid cell lines, one B- and one T-lymphoblastoid cell line (Namalwa and CEM, respectively), to produce MLV-based vectors. Upon transfection with a third generation packaging construct, the virus particle production by Namalwa cells was characterised by low RT-activity, and by CEM cells as high RT activity as previously established adherent packaging cells. An amphotropic packaging cell line (CEMFLYA) was therefore established from CEM cells. Upon introduction of a lacZ vector genome, the novel packaging cell line produced vector particles routinely in the region of 10^7 infectious units/ml. The vectors were helper-free and highly stable in fresh human serum. The potential for scaled up vector production was demonstrated by continuous culture of the new packaging cells for 14 days in a 250 ml spinner flask. These suspension packaging cells should be applicable to large bioreactor systems to bulk produce high titre, complement-resistant retrovirus vectors for gene therapy. Gene Therapy (2001) **8,** 737–745.

Keywords: *MLV; packaging; suspension cell; serum resistance*

Introduction

During the last two decades significant efforts have been made to develop packaging cells producing helper-free retroviral vectors for gene transfer. Investigations initially focused on the design of molecular constructs providing efficient expression of the viral packaging functions while preventing the generation of replication-competent viruses by recombination events with the vector component.[1–5] The early MLV packaging systems were based on murine fibroblasts, which are the cells more commonly used for the *in vitro* growth of MLV.[1–4] During the last few years, however, more attention has been paid to the choice of the cell type used to establish the packaging systems.[6,7] The results indicated that high titre production of retroviral vectors derives from a combination of both a construct efficiently expressing the helper functions and the choice of the cell type used. Two additional aspects of vector production are affected by cellular factors. First, the presence of endogenous retroviral sequences, particularly abundant in murine cells, raises safety concerns for the potential generation of replication-competent viruses from recombination events with the helper and vector constructs.[8,9] Second, high serum sensitivity is conferred to the viral particles by some cell lines. The sensitivity to the complement system is mainly determined by the acquisition of host components on the viral surface. The viral particles produced by nonprimate cells are inactivated by the human complement system mainly because they bear gal(alpha 1–3)gal terminal carbohydrates.[10–12] Screening of different cell types led to the identification of human cells which allowed the generation of high titre packaging cells producing serum resistant virus.[6,7] To date, studies on MLV vector production have been restricted to adherent cells.

Production of viral particles, such as bovine rhinotracheitis virus, reovirus, murine mammary tumour virus and human immunodeficiency virus, has been enhanced by the use of suspension cultures.[13–19] This has been achieved by the use of technologies such as roller flasks or microcarriers. However, a culture system using cells naturally growing in suspension may be preferable to one in adherence, when large-scale production of vectors is desired. For example, bulk production of viral vaccines is currently obtained by suspension cultures grown in large volumes and at high cell density by the use of perfusion-reactors and fermenters.[20,21]

In this study, a T-lymphoblastoid and a B-lymphoblastoid cell line were tested for their ability to produce MLV particles. A suspension amphotropic packaging cell line that produces high-titre, serum-resistant virus vectors was established and partially characterised in terms of optimum vector production and evaluated for cell growth and viral vector production.

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Results

Ability of lymphoblastoid cell lines to produce Gag proteins

MLV packaging functions were expressed using the plasmid CeB, in which a MoMLV LTR drives the expression of both *gag-pol* and a selectable marker, *bsr*.[7] The CeB plasmid was transfected into the B and T lymphoblastoid cell lines Namalwa and CEM, respectively, by electroporation. Transfectants were grown in the presence of the selective agent blasticidin S for 6 weeks before stable, drug-resistant populations (NamCeB, CEMCeB) were obtained. The cells were subsequently tested for production of virus particles. Cell supernatants from stable transfectants, parental cells and the high titre adherent packaging cell line, TELCeB6,[7] were subjected to ultracentrifugation and subsequent Western blot analysis using an antibody against Rauscher leukemia virus reverse transcriptase (RLV-RT). This antibody recognises Gag proteins, including $Pr65^{gag}$. The results showed that all samples derived from CeB transfectants, including the positive control TELCeB6 cells, contained similar amounts of Gag virus proteins (Figure 1). In addition to P30 (CA) protein, abundant high molecular weight Gag proteins could be detected in the viral pellet from NamCeB cultures, but not from CEMCeB or TELCeB6. This observation suggests the possibility that virus particles produced by Namalwa cells are less mature than those from CEM or TE671 cells.

CEMCeB cells produce high levels of RT activity

RT activity secreted by NamCeB and CEMCeB cells was evaluated and compared with that secreted by the high titre adherent producer cells TELCeB6. Cell free supernatants from CEMCeB, NamCeB and TELCeB6 cultures were serially diluted and analysed. RT activity values were normalised to that of the TELCeB6 cell supernatant (Figure 2). RT activity produced by CEMCeB cells was about 60% of that secreted by TELCeB6. In contrast, low RT activity was detected in NamCeB cell supernatant.

In order to select a cell population producing higher RT activity, 40 CEMCeB and 40 NamCeB single cell-derived clones were isolated from the bulk CeB transfectants and monitored for RT production. RT activity in supernatant of all NamCeB clones was less than 1/10 of the RT activity detected in the control supernatant of TELCeB6 (data not shown). These data confirm the inability of Namalwa cells to produce high titre of functional virus particles. In contrast, out of the 40 CEMCeB clones, 23 produced RT levels similar to that of TELCeB6 cells (data not shown). One clone, which in three repeated experiments produced the highest level of RT (CC31), was used to produce the full packaging cell line.

Efficient Env expression and incorporation into viral particles

The ALF and the AF plasmids, expressing the MLV 4070A envelope glycoprotein and the selectable marker *phleo*[7] were used to generate an amphotropic packaging cell line. While a potent C57LTR promoter drives the expression of the selectable marker in the ALF construct, *phleo* expression in the AF plasmid is suboptimal and relies on a translation reinitiation mechanism. ALF and AF were introduced into CC31 cells by electroporation. Transfectants were selected in the presence of phleomycin in order to produce stable populations expressing the amphotropic envelope glycoprotein. Stable resistant populations were readily obtained using the ALF, but not AF, plasmid. While additional studies would be required to obtain stable *env* expressing cells using the AF plasmid, we decided to further investigate the stable ALF transfectants (CC31A).

Efficiency of Env incorporation into viral particles was investigated. The viral pellets obtained by ultracentrifug-

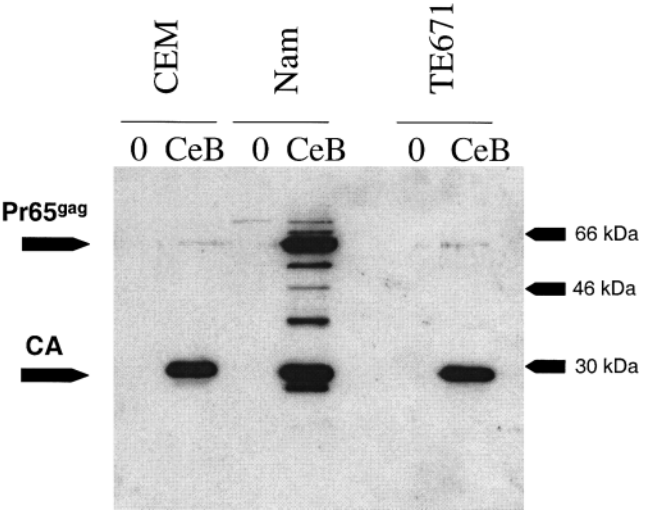

Figure 1 *Secretion of Gag proteins from CeB transfectants. Pellets were obtained from supernatants of CEM, Namalwa (Nam) and TE671 parental (0) and stable CeB transfectant (CeB) cells by ultracentrifugation and analysed by SDS-PAGE using a 12% gel followed by Western blotting and detection using anti-MLV RT antibody. The position of the molecular weight markers is shown. The presence of capsid (CA) and the full-length Gag ($Pr65^{gag}$) is indicated.*

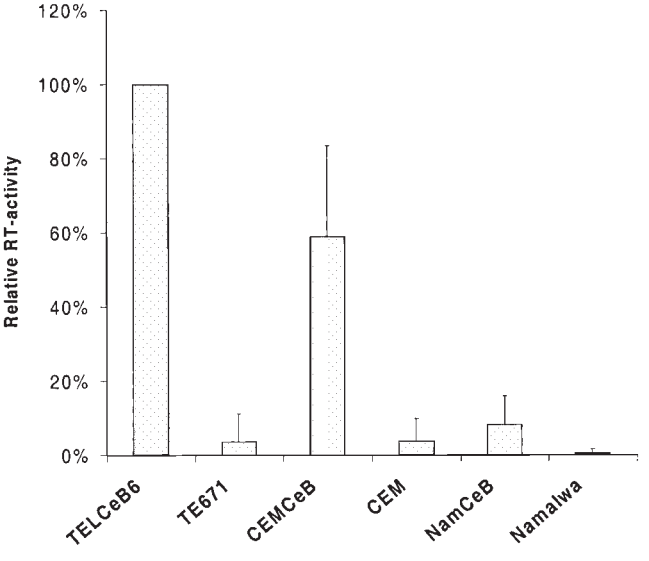

Figure 2 *RT activity released by stable CeB transfectants. RT activity was measured in cell supernatants using a colorimetric assay. RT activity measured in supernatants of Namalwa and CEM parental and stable CeB transfected cells are expressed as percentage of the RT activity measured in the supernatant of TELCeB6 packaging cells. Average values of three independent experiments are shown. Error bars represent standard errors of the mean.*

ation of cell supernatants were tested by Western blot analysis using a polyclonal antibody raised against the surface subunit of RLV Env (RLV SU). The SU was similarly detected in samples derived from CC31A and the positive control, TELCeB/AF-7 harbouring an Env expression plasmid AF[7] (Figure 3). Detection of P30 capsid proteins was performed in the same experiment and showed that a similar number of virions were analysed.

In order to select a cell population producing Env more efficiently, 40 single cell derived clones were isolated from CC31A cells. Env expression was first screened by FACS analysis of the different cell clones stained with anti-RLV SU and FITC-labelled secondary antibody. Assuming that the level of Env expression in the producer cells would correlate with the infectious titre, the four clones with the highest Env expression were identified. To compare their infectivity, MFGnlsLacZ, a retroviral vector expressing the reporter gene *LacZ* fused with a nuclear localisation signal,[22] was introduced into the four clones by transduction with helper-free MFGnlsLacZ pseudotypes bearing gibbon ape leukemia virus (GALV) Env.[23] The *LacZ* titre produced by the four different clones was evaluated on TE671 cells. All four clones produced a titre which was up to 10 times higher than the titre produced by the bulk CC31A population (Table 1). The clone CC31A11 was chosen and designated CEMFLYA.

In order to generate a packaging cell line producing a higher titre of MFGnlslacZ, 30 clones were isolated from CEMFLYAL cells, generated by transducing CEMFLYA with helper free MFGnlsLacZ(GALV) pseudotypes. The clone with the highest transduction activity was identified (CEMFLYAL19) (Table 2). CEMFLYAL 19 is a selected clone for all CeB, ALF and MFGnlslacZ constructs and therefore optimised to produce high-titre MFGnlslacZ vectors at a similar level as for TELCeB6/AF-7 cells.[7] The CEMFLYAL population and the CEMFLYAL 19 clone were further characterised.

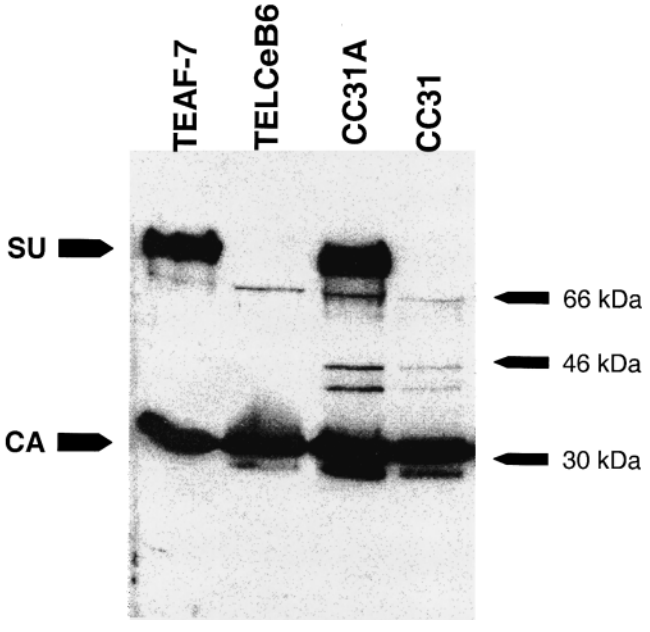

Figure 3 *Env incorporation in viral particles. Viral pellet from TELCeB6, CC31, TELCeB6/AF-7 and CC31A cell supernatants were prepared by ultracentrifugation and analysed by Western blot using anti-RLV gp69/71 and anti-RLV P30 antibodies.*

Table 1 *LacZ* titre produced by different *env* clones

Producer clone[a]	*Titre on TE671 (IU/ml)*[b]	
	First experiment	*Second experiment*
TELCeB6/AF-7	1.2×10^5	4.6×10^6
CC31A	1.8×10^4	ND
CC31A8	5.8×10^4	7.4 ± 10^4
CC31A11	7.4×10^4	3.7×10^5
CC31A15	2.4×10^4	ND
CC31A25	4.6×10^4	ND

[a]Pooled (CC31A) and clonal (CCA31A8, 11, 15 and 25) cell populations obtained by transfection of CC31 cells with the *env* expression plasmid, ALF, were transduced with MFGnlslacZ vector. Supernatants from bulk populations for MFGnlslacZ transduction were titrated on TE671 cells by *in situ* X-gal staining 48 h after infection.
ND, not done.

Table 2 *LacZ* titre produced by different MFGnlslacZ clones

Producer clone[a]	*Titre on TE671 (IU/ml)*
TELCeB6/AF-7	2.5×10^6
CEMFLYAL	1.8×10^4
CEMFLYAL1	1.1×10^5
CEMFLYAL9	5.7×10^5
CEMFLYAL19	2.2×10^6
CEMFLYAL20	1.0×10^6

[a]Supernatants from pooled (CEMFLYAL) and clonal (CEMFLYAL1, 9, 19, 20) cell populations obtained by transduction of MFGnlslacZ were titrated.

Helper free status of vectors produced by CEMFLYAL 19 cells

To test for the absence of replication-competent virus in CEMFLYAL 19 cells, we performed a vector mobilisation assay as described previously.[7] Supernatants derived from CEMFLYAL and 3T3LA cells were tested. 3T3LA cells harbour MFGnlsLacZ vector and are infected with wild-type MLV-A, so that they produce a mixture of replication competent MLV-A and MFGnlslacZ(MLV-A) pseudotypes.[24] Virus input of 10^7 and 10^3 *LacZ* infectious units (IU), respectively were plated on TEL indicator cells, which harbour the MFGnlsLacZ vector, but no MLV structural genes. After 2 weeks of culture, the supernatant of infected TEL cells was used to infect TE671 cells. Growth of a replication-competent virus in the indicator TEL cells would result in mobilisation and transfer of MFGnlsLacZ to TE671 target cells. No mobilisation of MFGnlslacZ could be detected for CEMFLYAL supernatant (Table 3), whilst the presence of helper-free virus was detected in the positive control supernatant derived from 3T3LA cells.

Serum sensitivity of virus produced by CEMFLYAL 19 cells

Resistance to the complement system of the virus produced by CEMFLYAL19 cells was tested. Viruses were harvested from CEMFLYAL 19 cells, murine 3T3LA cells

Table 3 Transfer of helper virus

Producer cells	*Input virus (IU/ml)*[a]	*Mobilisation of LacZ*[b]
CEMFLYAL	10^7	–
3T3LA	10^3	+++

[a]*LacZ* titre (IU) contained in virus input which was plated on TEL indicator cells is shown.
[b]Mobilisation of MFGnlslacZ vector from TEL indicator to TE671 target cells 2 weeks after original virus input on to TEL cells.
–, no *LacZ* infection; +++ *LacZ* infection at 10000–100000 IU/ml on TE671 cells.

and human FLYA4 lacZ 3 amphotropic packaging cells.[7] Viruses were exposed for 1 h at 37°C to human sera derived from three different individuals and infectivity was tested on human TE671 cells (Figure 4). The stability of virions was evaluated by comparison with the infectivity of inocula exposed to the same serum samples that had been heat inactivated. The virus produced by CEMFLYAL 19 cells was stable in fresh human serum, as the inoculum maintained 90% of the control infectivity after 1 h of treatment. In contrast, serum treatment reduced infectivity of 3T3LA-derived virus to less than 2%. CEMFLYAL 19 virus was even more resistant to human serum than FLYA4 lacZ 3 virus produced by human fibrosarcoma HT1080 cells. These data indicate that CEMFLYA produces vectors which are highly stable in the presence of human serum.

LacZ titre of virus produced by CEMFLYAL cells on different cell lines

The presence of cellular surface proteins in the viral envelope, acquired by the virus during egress from the producer cell, could confer on the vector a different adhesion and infectivity profile towards different cell types.[25–27] We therefore tested the possibility that vector virus infectivity may differ between suspension and adherent cell types. 1/20 dilutions of viral supernatant produced by CEMFLYAL cells (pooled population for MFGnlslacZ) and by TELCeB6/AF-7 cells were used to infect TE671, NIH 3T3, Raji and U937 cells. Two days after infection, the percentage of transduced cells was determined after X-gal staining. Viral infectivity was expressed as the percentage of transduced blue-staining cells (Figure 5). The results indicate that suspension cells as target cells were 10–20-fold less infectible with both viral preparations produced by suspension CC31AL cells and adherent TELCeB6/AF-7 cells. These data show that the two packaging cell lines produce vectors similar in target cell preference.

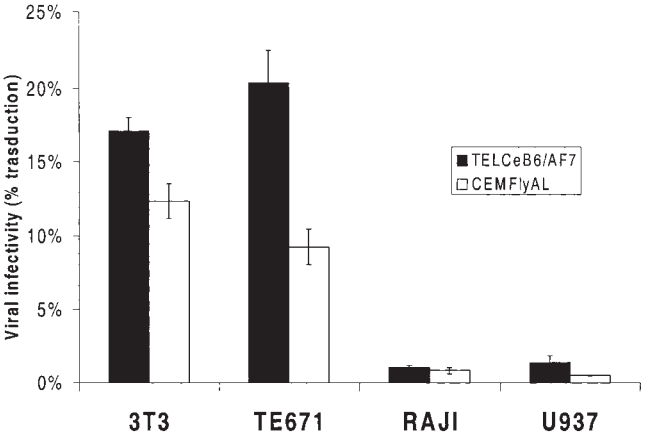

Figure 5 *Infectivity of CEMFLYAL and TELCeB/AF-7 virus on different cell lines. CEMFLYAL and TELCeB6/AF-7 viral supernatants were diluted 1/20 and used to infect subconfluent cultures of TE671, NIH 3T3, U937 and Raji cell lines. 48 hours after infection, percentages of transduced cells were determined. Values are average of triplicates. Error bars represent standard errors of the mean.*

Spontaneous decay of the retroviral vectors produced by CEMFLYAL 19 cells

It is known that retroviral vectors are rather unstable.[28–32] Reduction of *LacZ* titre of the vector produced by CEMFLYAL 19 cells in DMEM + 5% FCS was measured at different temperatures (Figure 6). The degradation constants calculated from the inactivation kinetics shown in Figure 6 indicate that the viral vector has half-lives of 3.9, 23 and 106 hours, when incubated at 37°C, 25°C, and 4°C, respectively. These results indicate that the vector produced from CEMFLYAL cells may be less stable than other vectors produced by some adherent packaging cells, such as ΨCRIP[30] and TEFLY (O Merten,

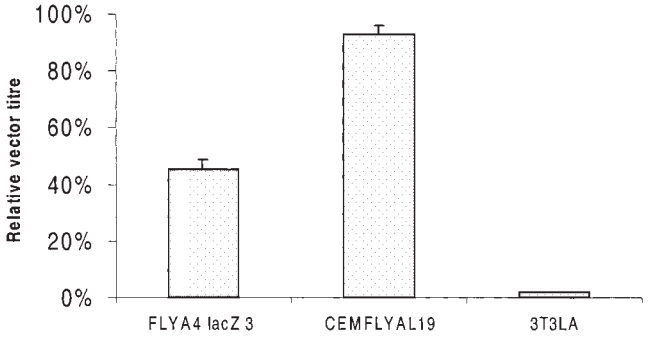

Figure 4 *Serum sensitivity of MFGnlslacZ(MLV-A) vectors. Virus supernatants from CEMFLYAL19, FLYA4 lacZ 3 and 3T3LA were mixed 1:1 with human sera from three different donors as previously described.[10] Relative LacZ titre after virus treatment with fresh sera was expressed as percentage of the titre after treatment with heat-inactivated sera. Average values of three independent experiments are shown. Error bars represent standard errors of the mean.*

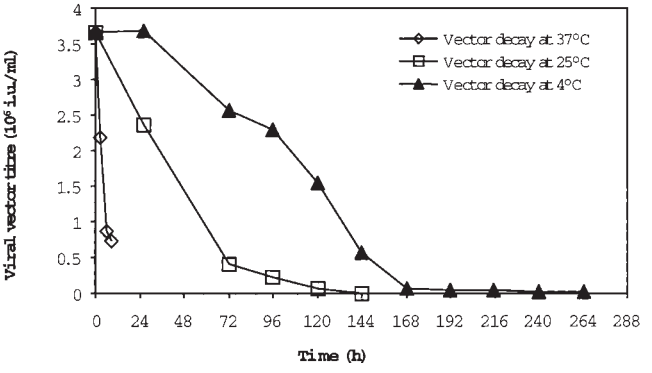

Figure 6 *Spontaneous decay of LacZ vectors from CEMFLYAL 19 cells. Virus supernatants harvested from CEMFLYAL 19 cells in DMEM + 5% FCS were incubated at 37°C, 25°C and 4°C and aliquots were taken and frozen at –80°C at regular intervals. LacZ titre was measured on TE671 cells.*

unpublished): half-lives of 7.5 h and of about 5 h at 37°C, of 53.3 h and of 61.3 h at 25°C, and of 268.8 h at 10°C and of 247.6 h at 4°C for ΨCRIP and TEFLY cell-derived vectors, respectively, were reported.

Effect of the culture conditions on virus titre: cell growth and vector production

CEMFLYAL 19 cells were grown in DMEM + 5% FCS as static cultures in T-flasks and in suspension in spinner flasks. The T-flask cultures allowed the production of up to 36×10^6 cells/ml, when the whole culture medium was changed twice a day. Under these conditions, up to 7.65×10^6 IU/ml (average titre: 2.44×10^6 IU/ml) were produced (not shown). Spinner cultures were started with 8×10^5 cells/ml (Figure 7). In order to harvest vector particles, as well as to change culture medium, 50% of the medium was changed every day for the period of day 1 to day 9, followed by a period of two changes per day (the first one in the morning, and the second one 8 h later; day 10–day 14). This switch in the frequency of medium change was necessary in order to sufficiently nourish the cells present in the culture (on day 9, 17.6×10^6 cells/ml were counted) and to keep the pH at an adequate level for cell growth and vector production (spinner flasks are not equipped with a pH controller). During this phase the cell density varied between 15 and 30×10^6 cells/ml, cell densities which cannot be supported with a single medium change per day. The following maximal vector titres were obtained: 1.5×10^7 IU/ml (average: 6.5×10^6 IU/ml for the period of day 1–day 9), and 1.9×10^7 IU/ml (average: 1.6×10^7 IU/ml for the period of day 10–day 14). Our spinner flask culture achieved 2.5–6-fold higher average titre than T-flask static cultures.

The comparison of the T-flask cultures and spinner flask cultures also revealed differences in the specific production rates. Whereas the rates varied from 0.4 to 2.02 IU/10^2 cell/h for the T-flask cultures, the spinner cultures produced one to five times more vector/cell/h. Depending on the frequency of medium exchange, the average specific production rate varied from 2.3 ± 1.4 (one 50% medium change every 24 h) to 5.3 ± 2.0 (one 50% medium change with 8 h interval) IU/10^2 cell/h (Table 4). This difference can be explained by more optimal culture conditions in spinner cultures than in T-flask cultures, because the spinner culture provides a homogeneous environment, whereas the T-flask culture system does not. Apparently a higher specific production rate was observed when the culture supernatant was changed after an 8-h interval, than when the change was carried out after a 16-h or 24-h interval, as practically the same absolute titres were obtained by 24-h, 16-h, or 8-h intervals. These differences can be explained by a relatively low stability of the retroviral vector at 37°C (see above). These results suggest that frequent medium change and vector harvesting, for example every 8 h, would be necessary to obtain high-titre, high-quality vector harvests, containing fewer inactivated vector particles.

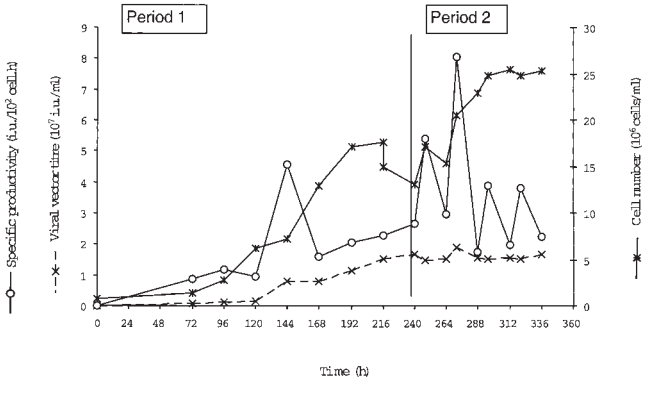

Figure 7 *Growth and viral vector production of CEMFLYAL 19 cells in spinner cultures. Spinner flasks were inoculated with 0.8×10^6 cells per ml. 50% of the medium was replaced by fresh medium daily up to 216 h (period 1), followed by a period of two medium changes of 50% per day (the first one at 9h00 and the second 8 h later, period 2). LacZ titre was measured on TE671 cells.*

Discussion

To date, there is no report of packaging cells for type C retrovirus vectors using suspension cells. In this report we investigated the ability of a T and a B cell line to produce retrovirus vectors and we describe the generation and partial characterisation of a packaging cell line based on T-lymphoblastoid cells.

We have observed a remarkable difference in the ability of the two suspension cell lines to produce functional MLV particles. While the transfection with a third generation packaging construct, CeB, led to production of high amount of Gag proteins and RT activity in CEM transfectants (CEMCeB), low RT activity was produced by Namalwa transfectants (NamCeB). Although NamCeB cells secreted as high amount of Gag proteins as CEMCeB and adherent TELCeB6 cells, significant amounts of high molecular weight Gag precursors were found in the virus pellet from NamCeB cells. These results suggest that NamCeB cells express less activity of two viral enzymes, protease and RT, encoded by the *pol* gene, compared with CEMCeB and TELCeB6 cells. The mechanism and cellular factors responsible for the inefficient expression of *pol* gene function in NamCeB cells is not known, while a possibility may be that read-through of Gag-Pol translation using a suppressor tRNA is inefficient. Other human B-lymphoblastoid cell lines may share this property of inefficient *pol* expression, as MLV particles produced by DG75 cells[33] containing CeB construct showed similar pattern to that of NamCeB cells in a Western blot using anti-MLV RT antibody (M Pizzato, unpublished). We selected CEM, but not Namalwa, cells for further development of packaging cells because of higher RT production by CEMCeB cells.

A clone expressing high levels of Gag-Pol and ampho-

Table 4 Range of titres and specific production rates for the long-term spinner cultures of CEMFLYAL 19 cells

Period (days)[a]	*Frequency of medium change (every h)*	*Range of titer (10^6 IU/ml)*	*Average specific vector production rate (U/10^2 cells/h)*
5–9	24	1.51–15.2	2.29 ± 1.37
10–14	16	15.0–16.6	2.22 ± 0.529
	8	14.8–19.0	5.27 ± 1.989

[a]The titres of the first days (day 0–day 4) were rather low due to the relatively low cell density.

tropic Env proteins was selected and named CEMFLYA. These cells are 'empty' packaging cells devoid of any vector construct. An MLV-based vector of choice could be introduced into these cells in order to produce infectious vector particles with a wide host range. Their ability to package a vector was demonstrated using the MFGnlslacZ vector. A clonal cell population (CEMFLYAL 19) selected after transduction of CEMFLYA cells with MFGnlslacZ produced *LacZ* titre varying between 3×10^5 and 8×10^6 IU/ml in static culture conditions. The virus titre is somewhat lower than that produced by currently available adherent cells (up to 10^8 IU/ml).[6,7]

CEMFLYA cells were selected for the high expression levels of both Gag-Pol and Env proteins. Although this procedure selected packaging cells producing the high end-point titre, it has recently been reported that an optimal ratio between P30 and Env expression may be required to maximise transduction efficiency at high multiplicities of infection.[34] Further optimisation of the viral protein levels and/or vector purification would be useful to maximise transduction efficiency.

Gag-pol and *env* constructs used for the generation of CEMFLYA have already been used in adherent packaging cells.[7] Such constructs have a reduced presence of unwanted virus sequences in order to minimise the risk of recombination and therefore generation of replication-competent virus. We tested the presence of RCR in virus supernatant containing 10^7 IU and did not detect any helper virus. This result confirms that generation of RCR when using CeB and ALF packaging constructs is low.[7] However, It should be noted that further improvement of packaging constructs in this safety aspect is possible. Constructs lacking MLV LTR are now available.[35]

Serum sensitivity of virus particles is greatly affected by the presence of gal(alpha 1–3)gal terminal carbohydrates on the virus envelope originating from non primate cells.[11,12] It was therefore expected that virus originating from CEM cells would have been relatively resistant to the complement activity. Exposure of CEMFLYA virus to human fresh serum impaired virus infectivity by only 10%. This result shows that CEMFLYA virus is very stable and could be a good candidate for *in vivo* applications.

A possible reason for the lower sensitivity to MLV infection of suspension cells, such as lymphoblastoid cells, is their poor ability to adsorb virus particles on to their membrane, despite relevant expression of the specific virus receptor.[25] We tested whether the virus produced by CEMFLYA have a different infectivity for suspension cells, compared with that from adherent TELCeB6/AF-7 cells, and found no preferential tropism of CEMFLYA virus to suspension cells. Accordingly, we observed effective virion binding on adherent target cells, but not on suspension target cells, for both viruses (data not shown).

Retrovirus vectors are relatively unstable during their preparation. Our analysis of thermal stability of the vector produced by CEMFLYA cells showed that they have a rather short half-life, 4 h at 37°C, and may be even less stable than vectors produced by some other retrovirus packaging cell lines. Vectors produced by CEMFLYA cells therefore should be stored at 4°C immediately after production and the downstream-processing started as soon as possible. Conditions of vector preparation avoiding the rapid degradation of CEMFLYA-derived vectors should be pursued in future.

The technological advantage of the use of retrovirus packaging cells based on suspension culture is that these cells grow to high cell density in a culture system with almost unlimited potential for scale-up. The production systems generally used for anchorage-dependent cells are those providing large surfaces. Apart from the fluidized bed reactor[36,37] and the microcarrier culture systems,[38] which have a large scale-up potential, all other reactor systems developed for surface attached growth (CellCube, hollow fiber system, fixed bed reactor system) are limited in their scale. The use of suspension culture systems (stirred tank reactor system) has the main advantage of an almost unlimited potential of scale-up which is proven in theory and practice.[39,40]

Although most of the adherent cell lines can be adapted to suspension growth (as single cells or aggregates), the vector titres produced by such adapted cells can be negatively affected, and such an adaptation has rarely been reported. For instance, the adaptation of TEFLY cells to growth in suspension in standard culture medium led to the formation of huge clumps which showed reduced growth and a reduction in the vector production by a factor of 10–100 when compared with cultures in packed bed reactors.[41] The only cell line successfully adapted to suspension growth was the HEK293 based ProPak packaging cell line, producing between 2×10^7 and 4×10^7 IU/ml in suspension as well as in packed bed reactor cultures (SP Forestell, personal commununication). In this study, the suspension culture of CEMFLYAL 19 cells at cell densities of 10–20 $\times 10^6$ cells/ml lead to similar vector titres as observed for the TEFLY GA and TEFLY A cell lines,[41] as well as to those obtained for ProPak cells grown in suspension or packed bed cultures (SP Forestell, personal communication). These results indicate that CEMFLYA-derived packaging cells would be ready to be applied to larger scale production systems, such as perfusion reactors. Preliminarily, we have obtained a vector harvest of 5×10^6 IU/ml by a 1.4 litre reactor used in perfusion mode. It is expected that much higher titres will be possible in optimised reactor cultures.

In conclusion, we have developed a retrovirus packaging cell line based on a suspension lymphocytic cell line. This packaging cell line can produce high-titre, helper-free, serum-resistant vectors and should be easily applicable to large-scale production systems.

Materials and methods

Cells and viruses

Human Namalwa, Raji, U937 and CEM (CEM-CCRF) cells and their derivatives were grown in RPMI1640 medium (RPMI, Gibco BRL, Paisley, UK) unless otherwise indicated. Murine NIH 3T3, human TE671 and HT1080 cells and their derivatives were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL). Growth media were supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), penicillin G (100 U/ml) and streptomycin (100 μg/ml) and cell cultures were maintained at 37°C and 5% CO_2. TELCeB6, TELCeB6/AF-7 and TELCeB6/GAF are TE671-derived packaging cells producing helper free, MLV-based retro-

virus vector particles which encode the MFGnlslacZ genome and bear no MLV-A and GALV envelope glycoproteins, respectively.[7,23,42] A HT1080 based packaging cell line, FLYA4 lacZ 3 was also used.[7]

Antibodies

Goat polyclonal anti-MLV RT, anti-RLV P30 and anti-RLV gp69/71 (Quality Biotech Inc, Camden, USA) were used to detect MLV Gag and Env proteins. Preliminary Western blot analyses using anti-MLV RT and anti-RLV P30, indicated that anti-MLV RT cross-reacts to MLV P30 as well as Gag precursor proteins with a similar pattern to that of anti-RLV P30. Fluorescein isothiocyanate (FITC)-labelled donkey IgG against goat IgG (Jackson, West Grove, PA, USA) was used as secondary antibody in FACS experiments. HRP-labelled anti-goat antibody (Sigma, Poole, UK) was used as secondary antibody in Western blot assays.

Transfection and isolation of resistant colonies

Gag-pol and *env* constructs were separately introduced into Namalwa and CEM cells by electroporation. 8×10^5 cells/ml culture were incubated overnight in fresh medium. Cells were centrifuged at 325 *g* for 5 min and resuspended in fresh and pre-warmed medium at the final concentration of 10^7 cells/ml. 1 ml of cell suspension was mixed with 10 μg of plasmid DNA and transferred into a 0.4-cm electrode gap cuvette for electroporation (BioRad, Hemel Hempstead, UK). Samples were electroporated using a Gene Pulser II electroporator (BioRad) at a voltage of 250 mV and a capacitance of 975 μF. Immediately after electroporation, cells were resuspended in 10 ml of fresh pre-warmed medium. After 24 h, cells were cultured from a starting concentration of 5×10^5 cells/ml in fresh medium supplemented with the appropriate concentration of selective agent; 6 μg/ml blasticidin S for the gag-pol construct, CeB and 50 μg/ml phleomycin for the *env* construct, ALF. Culture medium was replaced with fresh selection medium every 5 days and after 4–6 weeks resistant cell populations were obtained.

From the cell populations after selection, cell clones were isolated by limiting dilution. Nonreplicating feeder cells were used to support the growth of single cells during the isolation of clones of CEM and Namalwa cells. 1×10^8 CEM or Namalwa cells were pelleted at 325 *g* for 10 min, resuspended in 1 ml of culture medium and X-irradiated with 40 Gy at 5 Gy per min. Cells were aliquoted and frozen for later use. Absence of cell growth was verified by culturing the cells for 3 weeks from a starting concentration of 1×10^6 cells/ml. Cells harbouring CeB or ALF plasmids were incubated at 8×10^5 cells/ml overnight in fresh medium. Cells were plated on to U bottom 96 well plates at a concentration of 0.1, 1 and 10 cells per well with 200 μl of fresh medium and 10^4 autologous feeder cells per well. Colonies were harvested 4–5 weeks later, and transferred into 24-well plates in fresh selection medium from plates where no more than 20 wells presented cell growth.

Introduction of MFGnlslacZ vectors into CEM-derived packaging cells

CEM cells and their derivatives were cocultivated with TELCeB6/GAF cells, which produce helper-free MFGnlsLacZ pseudotypes bearing GALV Env,[23] for 10 days. Adherent TELCeB6/GAF cells were depleted by several passages of suspension cells in tissue culture flasks until no adherent cells were present. More than 95% of suspension cells became *LacZ*-positive.

Virus production

Adherent producer cell lines cultivated in T-flasks: MLV production from adherent producer cells was performed by maintaining confluent cultures for 2 days in fresh medium. The medium was then changed, harvested after 24 h and filtered through 0.45 μm filters (Sartorius, Goettingen, Germany).

CEMFLYAL 19 cells cultivated in T-flasks and in spinner flasks: Virus production from suspension producer cells in T-flasks was performed by culturing the cells in fresh medium for up to 24 h. The sample was centrifuged at 325 *g* for 10 min and supernatant was filtered through a 0.45 μm filter. Spinner flasks (Techne, Cambridge, UK, Ref. F7609) were inoculated with 250 ml of a cell suspension at 0.8×10^6 cells/ml. The cultures were performed in DMEM supplemented with 5% of FCS. The pH was adjusted by injection of CO_2. The spinners were incubated at 37°C using an agitation of 30 r.p.m. Samples of the cell suspension were taken for viability assessment by cell counting using trypan blue. In order to replace the medium, cells were pelleted at 1500 r.p.m. (458 g) for 5 min. After centrifugation, culture supernatants were taken for the virus titration. The cell pellet was resuspended in fresh medium and reintroduced into the spinner flask.

Growth and vector production kinetics of CEMFLYAL 19 cells in spinner cultures

Cultures in spinner flasks were started with 0.8×10^6 cells per ml and treated as described previously. 50% of the medium was replaced by fresh medium daily over the first 216 h (period 1). At 216 h, the cell density was reduced to 15×10^6 to avoid a too rapid increase of the cell density leading to a deterioration of the culture conditions and to permit further observation of the culture. During period 2 (after 216 h), 50% of the medium was changed twice a day, the first change at 9h00, and the second one 8 h later. At each medium change, supernatant samples were taken for virus titration and cells were sampled for the determination of the cell density. The cell-specific vector production rate (SVPR IU/cells/h) was calculated as:

$$SVPR = (V2 - V1 \times 0.5) \times 2/((C2 + C1) \times (t2 - t1))$$

whereby, V1, virus titre (IU/ml) at sampling point t1; V2, virus titre (IU/ml) at sampling point t2; C1, cell number per ml at sampling point t1; C2, cell number per ml at sampling point t2; t1, t2, sampling points in h.

Infection assays

Adherent target cells (TE671) were seeded the day before infection at a concentration of 5×10^4 cells in 0.5 ml per well of a 24-well tissue culture plate. Cells were incubated with 0.5-ml dilutions of virus supernatant for 4 h at 37°C before the virus inoculum was replaced with fresh medium. Suspension target cells were seeded the day before infection at a concentration of 2×10^5 cells/ml in 2 ml per well of a six-well plate. Virus dilutions were added in ratio of 1:1 to the suspension cultures and cells

were cultured for 4 h at 37°C. Plates were then centrifuged at 325 *g* for 10 min and virus supernatant was replaced with fresh medium.

After 48 h, cells were stained using X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside) as previously described.[10,43] Using adherent target cells, clusters of stained cells were attributed to a single infection event and titre was expressed as *LacZ* infectious units (IU) per ml of virus supernatant. When transduction efficiency was determined, percentage of transduced cells was determined by counting the number of stained cells over a total of 1000 cells in randomly chosen microscopy fields.

Serum sensitivity

The stability of *LacZ* pseudotype viruses in fresh human serum was examined by titrating surviving virus after incubation in a 1:1 mixture of virus harvest in serum-free medium and fresh human serum for 1 h at 37°C as described before.[10]

Vector mobilisation assay

Vector mobilisation assay to detect replication-competent viruses was carried out following the method described previously.[7] Virus particles were concentrated by overnight low speed centrifugation at 2000 g of CEMFLYAL19 cells.[44] The pellet produced by centrifugation of 50 ml of virus suspension was resuspended in 100 μl and titrated for MFGnlsLacZ on TE671 cells. Transduction efficiency was linear to input volume, indicating that inhibitors of transduction present in cell supernatant were eliminated by centrifugation (data not shown). 10^6 TEL indicator cells, which are TE671 cells containing the MFGnlslacZ vector, but no MLV structural genes, were challenged with the concentrated virus from CEMFLYAL 19 (10^7 *LacZ* IU) or a dilution of virus supernatant containing helper positive MFGnlslacZ(MLV-A) produced by 3T3LA cells (10^3 LacZ IU).

Western blot

Six ml of virus supernatant was harvested from a confluent T25 flask of adherent producer cells in DMEM supplemented with 2% FCS or from a culture of 12×10^6 suspension cells in RPMI 2% FCS. Supernatant was filtered through a 0.45 μm filter and pelleted by centrifugation at 100000 *g* in a SW40 rotor (Beckman, High Wycombe, UK) for 1 h at 4°C. The virus pellet was resuspended in 200 μl of PBS and stored at –80°C.

Fifteen μl of each sample were mixed 1:1 with gel loading buffer (100 mM Tris pH 6.8, 4% SDS, 20% glycerol, 5% 2-β mercaptoethanol, 0.02% bromophenol blue), boiled for 3 min and run on 12% polyacrylamide gels. Proteins were transferred on to nitrocellulose membrane (Amersham, Bucks, UK). The blots were washed in Tris buffered saline (TBS; 25 mM Tris-Base pH 7.4, 140 mM NaCl, 5 mM KCl), 0.1% Tween 20 (TBS-Tween) for 10 min and blocked in TBS-Tween containing 5% non-fat milk powder (TTM) for 1 h at room temperature. Membranes were incubated with 1:1000 goat anti-RLV SU and 1:5000 goat anti-RLV CA or goat anti-MLV RT in TTM for 1 h at room temperature, washed three times in TBS-Tween and incubated with 1:5000 HRP-labelled anti-goat antibody in TTM for 1 h at room temperature. After the membrane was washed three times with TBS-Tween and once with TBS, protein detection was performed using the enhanced chemiluminescence system (ECL, Amersham) according to the manufacturer's instructions and using Kodak Biomax MR film.

RT assay

RT activity from virus supernatants was measured using an RT assay kit (Type C RT assay; Cavidi Tec, Uppsala, Sweden) following the manufacturer's instructions. Standard concentration of recombinant MLV RT were used to build a linear dose–effect curve.

FACS analysis

Cells were collected by centrifugation at 325 *g* and washed three times with ice cold phosphate buffered saline (PBS) with 1% BSA (bovine serum albumin) and 0.1% sodium azide (PBA). Cells were incubated with 1:2000 dilution of anti-RLV GP69/71 antibody, washed three times with PBA and incubated with the secondary antibody for 45 min at room temperature. After a final wash, samples were analyzed by FACScan (Becton Dickinson, Oxford, UK).

Stability assays

CEMFLYAL 19 supernatant was produced in two 162 cm^2 T-flasks, inoculated with 25 ml at 7×10^6 cells per ml. Two days later, the whole medium was replaced by fresh medium. On the following day, the cell suspension was centrifuged and the supernatant collected in 75 Eppendorf tubes (1.5 ml), in 0.5 ml aliquots. Six tubes were immediately frozen at –80°C, nine stored at 37°C (incubator), 30 tubes at 20°C and 30 tubes at 4°C. Three tubes each were taken and frozen at –80°C every 3 h for the samples stored at 37°C, or at 24-h intervals for 2 weeks for those stored at 20°C and 4°C. After termination of the test, titrations were performed in order to determine the decay of the titre. Decay rate constants and virus half-lives were determined by regression calculations based on an exponential regression for the 37°C assays and on a linear regression for the tests performed at 25°C and 4°C.

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