Relationship of Endogenous Murine Xenotropic Type C Virus Production to Spontaneous Transformation of Cultured Cells

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SUMMARY

Spontaneous production of endogenous xenotropic viruses by clones of New Zealand Black (NZB) embryo cells occurs at a constant level even after several cell transfers. Foci of cell alteration occur spontaneously in some of the clonal lines after 6 to 10 passages. The amount of virus generated does not correlate with spontaneous transformation of these NZB cells nor of cells from NIH Swiss or BALB/c mice. The data demonstrate an intracellular regulation of xenotropic virus expression which remains stable over several cell generations and differs from that controlling cell transformation.

INTRODUCTION

Murine xenotropic (X-tropic) viruses are a distinct class of endogenous murine type C viruses (MuLV) which are unable to productively infect mouse cells. X-tropic viruses can be propagated in cells from heterologous species whereas endogenous ecotropic MuLV can infect and spread in mouse cells (Gross, 1970; Levy & Pincus, 1970; Levy, 1973, 1974).

Many, if not all, strains of mice harbour xenotropic viruses but the virus is not expressed to a similar extent in all strains. New Zealand Black (NZB) mice represent an unusual case in that all cells examined from this mouse strain from embryo to adulthood produce the virus spontaneously and at relatively high titres (Levy et al., 1975). NIH Swiss and C57 BL/6 mice produce the virus in moderate amounts, whereas 129/J and SWR produce no detectable replicating X-tropic virus (Levy, 1975). Thus, control of xenotropic virus gene expression clearly varies between mouse strains and is at a minimum in NZB mice. Cells derived from different tissues of NZB mice also produce variable quantities of infectious virus (Levy et al. 1975). This observation suggests that the intracellular control of X-tropic virus production can vary from cell to cell even within the NZB strain.

Using lines established from single cell clones of NZB embryo cells, we have studied the regulation of spontaneous production of the NZB virus. The interaction between endogenous virus production and the ability of clones to transform spontaneously has also been examined.

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METHODS

Cells and virus. NIH Swiss and BALB/c mouse embryo (ME) cells were supplied by Microbiological Associates, Bethesda. NZB mouse embryo cells were prepared in this laboratory from 15-day-old embryos obtained from pregnant NZB mice supplied by Jackson Laboratory, Bar Harbor. The NZB-Q cell line was obtained from Ms Marilyn Lander, Bethesda. Single cell clones of the NZB-Q embryo cell line were prepared as described previously (Levy et al. 1975). BALB/3T3 (Jainchill et al. 1969), NIH/3T3 (Todd & Green, 1963) and dog osteosarcoma (D17) cells were provided by Drs W. Nelson-Rees, Oakland and P. Arnstein, Berkeley. The normal rat kidney (NRK) cell line (Duc-Nguyen et al. 1966) and the NRK-Harvey line (Levy, 1971) have been maintained in this laboratory. The NRK-Harvey line is an NRK cell line non-productively transformed by the Harvey strain of murine sarcoma virus (H-MSV). Clone B-4 derived from this line was employed for the virus rescue experiments (Levy et al. 1975). All cultured cells, except the mink S+L- cell line (Peebles, 1975), were maintained in Eagle's minimal essential medium (Grand Island Biological, Gibco, New York) containing: 10% unheated foetal bovine serum (FBS), 2 mM-glutamine, 250 μg/ml streptomycin and 250 units/ml penicillin. For focus formation assays measuring MSV, the FBS was replaced by 5% heated (56 °C for 30 min) calf serum.

Co-cultivation assays. Mouse cells were passaged in flasks such that they were always subconfluent. For the co-cultivation assay at each passage level the cells were plated at 2.0 × 10^5 cells per 6 cm Petri dish. When cells reached 50% confluence (usually 48 h later) the medium was changed and clone B-4 NRK-Harvey cells were added to half the cultures. All cultures were subsequently refed with medium on days 5 and 7. On day 9, fluids were removed and retained for focus or immunofluorescence assays as described below. By day 9, the B-4 cell overlay was at least 90% confluent; plates containing only the mouse embryo cells were fully confluent by this time and were examined for foci of altered cells. Following completion of these procedures all plates were irradiated and co-cultivated with XC cells for detection of ecotropic MuLV as described by Rowe et al. (1970).

Focus formation assays. NRK cells were plated at 3 × 10^5 cells per 6 cm Petri dish in maintenance medium. Twenty-four hours later these cells were treated with DEAE-dextran, 25 μg/ml for 30 min at 37 °C, to increase their sensitivity to virus infection (Duc-Nguyen, 1968). After removal of dextran, the virus inoculum (0.4 ml) was allowed to adsorb for 30 min at 37 °C. The cultures were then refed with medium which was subsequently changed every 3 days. Foci were counted on days 7 and 9. A known quantity of MSV was included in each assay in order to correct for any variation in the sensitivity of the NRK monolayer cells. In practice, no variation was observed except in one assay in which the number of foci was half that expected.

Immunofluorescence assay. Dog osteosarcoma cells were plated in Petri dishes containing three glass coverslips (11 × 22 mm). Twenty-four hours later, the cells were treated with DEAE-dextran for 30 min and then inoculated with filtered cell culture supernatants (0.4 ml) as described above. Six days later, the coverslips were removed, washed and fixed in cold acetone and then stored at -70 °C. They were examined for foci of cells with cytoplasmic fluorescence using a fluorescein isothiocyanate conjugated goat antiserum (supplied by R. E. Wilsnack, Huntington Research Laboratories) prepared against Tween-ether disrupted Moloney MuLV (Hartley & Rowe, 1976). This antiserum identified the group-specific (gs) MuLV antigen.
Table I. Yields of NZB pseudotype sarcoma virus after co-cultivation of NZB cell clones with NRK-Harvey cells*

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Clone 1 (passage 10)</th>
<th>Clone 35 (passage 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$3.7 \times 10^4$</td>
<td>$3.7 \times 10^4$</td>
</tr>
<tr>
<td>2</td>
<td>$3.8 \times 10^4$</td>
<td>$4.0 \times 10^4$</td>
</tr>
<tr>
<td>3</td>
<td>$3.8 \times 10^4$</td>
<td>$3.8 \times 10^4$</td>
</tr>
<tr>
<td>4</td>
<td>$3.0 \times 10^4$</td>
<td>$4.0 \times 10^4$</td>
</tr>
</tbody>
</table>

* Cells of each clone were co-cultivated with the B-4 non-virus producing MSV-transformed cell line. The day 9 supernatants were filtered and assayed for focus formation on NRK cells as described in Methods. Titres are expressed as focus forming units (f.f.u.)/ml.

Those cultures with no foci of fluorescence were transferred up to six times to ascertain the possible presence of virus. After three transfers no change in titre occurred. The titre per Petri dish was calculated and expressed as ‘fluorescence units’ (FU)/ml.

Mink S+L− assay. The quantity of xenotropic MuLV produced by NZB mouse embryo cells was also measured by the S+L− mink cell assay (Peebles, 1975). Filtered culture supernatants were inoculated on to the mink cells plated 24 h previously in polybrene (1 μg/ml). After adsorption of the supernatant for 30 min at 37 °C, the cells were refed and maintained in Dulbecco’s modified medium containing polybrene (1 μg/ml). Foci of cell alteration were first noted at 6 days and final readings were made at 10 days.

RESULTS

Reproducibility of the co-cultivation assay for detection of spontaneous xenotropic virus production

Spontaneous production of X-tropic virus by clones of NZB-Q cells at a given passage level was measured by the co-cultivation assay on several occasions. Previous studies indicated that the extent of MSV rescue directly correlated with the relative amounts of infectious X-tropic viruses produced by the cells (Levy et al. 1975). Two high producing clones of the NZB line (C1 and C35), at passages 10 and 5 respectively, were co-cultivated in quadruplicate with B-4 cells, and the supernatants were assayed for focus forming virus in NRK cells. These experiments showed that the co-cultivation assays were highly reproducible (Table 1).

Since errors in cell counting or variation in cell viability from week to week might cause fluctuations in cell numbers, the effect on the final titre of pseudotype virus produced which was caused by varying the number of cells plated was investigated. The results indicated that halving the initial number of cells did not seriously affect the titre of virus obtained with co-cultivation of clone 1 or clone 35 cells (Table 2). Confluence of both cell lines was clearly achieved well in advance of the final fluid harvest. In contrast, increasing the number of cells plated from the NZB-Q clone 28 line, from a very low value, caused a dramatic decrease in virus titre. Microscopic observation showed that the B-4 cells were unable to adhere to C28 cells and remained in the culture only if they directly attached to the bottom of the Petri dish. These experiments indicated that it is important to ensure that the cells being assayed for virus permit an overlay of the indicator NP cells. This criterion was met for all the cells examined except for clone 28.
Table 2. Effect of variations in the initial number of cells plated on the titre of NZB pseudotype sarcoma virus produced by co-cultivation*

<table>
<thead>
<tr>
<th>Clone</th>
<th>Number of cells plated (f.f.u./ml)</th>
<th>Virus titre (f.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>$1 \times 10^5$</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td>2</td>
<td>$2 \times 10^5$</td>
<td>$7 \times 10^4$</td>
</tr>
<tr>
<td>35</td>
<td>$1 \times 10^5$</td>
<td>$3 \times 10^4$</td>
</tr>
<tr>
<td>35</td>
<td>$2 \times 10^5$</td>
<td>$4 \times 10^4$</td>
</tr>
<tr>
<td>28</td>
<td>$2 \times 10^4$</td>
<td>$2 \times 10^3$</td>
</tr>
<tr>
<td>28</td>
<td>$5 \times 10^4$</td>
<td>$2 \times 10^4$</td>
</tr>
</tbody>
</table>

* See Table 1 for procedure.

Table 3. Effect of passage of the NZB clonal lines on the quantity of xenotropic pseudotype virus produced during co-cultivation*

<table>
<thead>
<tr>
<th>NZB clone</th>
<th>Starting passage no. (week 1)</th>
<th>Xenotropic virus titre (f.f.u./ml) after week:</th>
<th>Mean titre (f.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>$4 \times 10^4$ $4 \times 10^4$ $2 \times 10^4$ $5 \times 10^4$ ND</td>
<td>$4 \times 10^4$</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>$5 \times 10^2$ $8 \times 10^2$ ND</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>7</td>
<td>$3 \times 10^3$ $6 \times 10^3$ $4 \times 10^3$ $4 \times 10^3$ $4 \times 10^3$</td>
<td>$4 \times 10^3$</td>
</tr>
<tr>
<td>35</td>
<td>5</td>
<td>$4 \times 10^4$ $3 \times 10^4$ $6 \times 10^4$ ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Virus production following co-cultivation of NZB cells with the NRK-Harvey line was measured as described in Table 1.
† ND = Not determined.

Variation in X-tropic pseudotype virus production by different clones of NZB cells after several passages

Four clones isolated from the NZB-Q cell line were co-cultivated with B-4 cells and assayed for xenotropic pseudotype virus production. The co-cultivation procedure and virus assays were repeated after weekly transfers of the cells for up to 5 weeks.

Two conclusions were drawn for the data obtained (Table 3). (1) The titre of virus produced varied from clone to clone with a difference of 100-fold between cells producing the lowest and highest quantities of infectious virus. (2) The quantity of the X-tropic virus pseudotype of MSV produced by each cell clone during co-cultivation was essentially constant and did not change with repeated cell transfers.

All cells, alone or after co-cultivation with the B-4 line, were assayed for ecotropic virus by the XC test (Rowe et al. 1970) and no plaques were detected. This observation confirmed previous reports that NZB cells do not release ecotropic virus, and that xenotropic viruses cannot form syncytia in XC cells, even when present in high titre (Levy 1973, 1974). We have observed that this lack of syncytium formation occurs despite efficient replication of X-tropic viruses in XC cells. In addition, the negative results from the co-cultivated cells show that the rescued MSV genome is also not able to cause syncytia formation in XC cells.

Detection of spontaneous X-tropic virus production by NZB cells using immunofluorescence and S+L- mink cell assays

Using the immunofluorescence test, assay of xenotropic virus production was as reproducible as by the co-cultivation technique and confirmed the variation in spontaneous X-tropic virus production by the NZB clones (Table 4). The most accurate measurement of
Table 4. Spontaneous production of xenotropic viruses by clones of NZB cells

<table>
<thead>
<tr>
<th>Virus titre</th>
<th>Immunofluorescence assay (FU/ml)†</th>
<th>S + L− assay (f.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone</td>
<td>P₁</td>
<td>P₃</td>
</tr>
<tr>
<td>35</td>
<td>$3 \times 10^9$</td>
<td>$2 \times 10^4$</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>$1 \times 10^5$</td>
</tr>
</tbody>
</table>

* The quantity of virus produced by the clones was determined by indirect immunofluorescence tests on dog D17 cells and by mink S+L− assays as described in Methods. Included for the immunofluorescence studies are the virus titres measured in the dog cells 1 week after virus co-cultivation (P₁) and after three transfers of the cells (i.e. 3 weeks, P₃).

† Fluorescence units/ml.

virus titre required passage of the indicator monolayer cells for 3 weeks (Table 4) and indicated that co-cultivation amplified the detection of virus production four- to fivefold.

No group-specific (gs) antigen was detected in the low virus producer, clone 4, and high virus producer, clone 35. As spontaneous virus production occurs in these cells, this result (confirmed by J. Riggs, Berkeley) is surprising. Either levels of the gs antigen are too low to be measured, or the antigen is expressed in a form not detectable by the assay.

The quantity of virus spontaneously produced by the NZB clones was also measured by the mink S + L− assay. Clone 35 had a titre of $4 \times 10^2$ f.f.u./ml and clone 4, 10 f.f.u./ml (Table 4). This assay therefore also demonstrated the difference in production of virus by two different NZB cells, but was less sensitive for measuring X-tropic virus than co-cultivation or immunofluorescence. Recent evidence suggests that the S + L− assay may be measuring a different NZB-MuLV than immunofluorescence tests (O. Varnier & J. A. Levy, unpublished observations; R. Chussed, J. W. Hartley & W. P. Rowe, personal communication).

Spontaneous transformation of NZB cell clones

Clones of cells isolated from the established NZB-Q mouse embryo line (Levy et al. 1975) were selected on the basis of having an epithelial cell morphology as close as possible to that of non-transformed cells (Fig. 1). Because of the evidence that mammalian cells infected with virus may transform more readily than uninfected ones (Freeman et al. 1970, Rhim et al. 1971), we wished to determine whether cells producing X-tropic virus retained this near normal morphology on repeated passage. At each transfer, cells were allowed to reach confluence after plating and were observed for the appearance of foci of transformed cells.

All of the NZB clones examined developed foci (Fig. 1) but differed in the extent of this spontaneous transformation. To measure the rate of onset of transformation and the proportion of the cells transformed, foci were counted at each passage. The mean results of duplicate determinations are presented in Table 5. All clones first showed areas of transformation between passages 7 and 11 since initial cloning. However, the degree of transformation varied from almost complete involvement of all cells (e.g. clone 1) to just 1 focus per plate (clone 35). No focus forming viruses were detected in cultures that spontaneously transformed. This alteration in cell morphology did not correlate with the extent of X-tropic virus production (see Table 3). Moreover, the level of virus production by the cell clones before and after development of foci did not differ (data not shown).
Fig. 1. (a) Clone 1 of the NZB-Q cell line. Note the cobblestone epithelial appearance of this monolayer. (b) Spontaneous foci of cell alteration in the clone 1 cell line. (c) Spontaneous foci in the clone 4 cell line. (d) Spontaneous foci in the clone 9 cell line. Magnification ×40.
Xenotropic viruses and cell transformation

Table 5. Spontaneous transformation of NZB-Q cell clones after multiple passages

<table>
<thead>
<tr>
<th>NZB cell clone</th>
<th>Cell passage first showing</th>
<th>Number of foci per plate; increasing passage number →*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TNC†</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>30</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>35</td>
<td>7</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* The number of foci were recorded at the first passage showing transformation and at subsequent consecutive passages.
† TNC = Too numerous to count.
‡ ND = Not determined.

Characterization of subclones of the NZB embryo cell clones

Virus production by any clone of the NZB-Q line could be an average value for all cells present or represent a characteristic production by every cell derived from the same parental cell. In order to examine these possibilities and to determine directly whether virus production differs between transformed and non-transformed clonally related cells, clones 1 and 4 were subjected to a second cycle of cloning. Cells with normal or transformed morphology were selected. The subclones were assayed for virus production by the co-cultivation assay. The results (not shown) indicated that virus production was the same for all the subclones derived from clone 1. A completely transformed subclone of clone 1, did not differ in its extent of virus release. In addition, virus production by a transformed subclone of clone 4 was identical to that obtained with the parental clone 4 cell line.

Behaviour of NIH/3T3 and BALB/3T3 cells on repeated passage

In other attempts to correlate cell transformation with xenotropic virus expression, NIH/3T3 and BALB/3T3 lines were chosen as controls. They resembled the NZB-Q line in that they were derived from mouse embryos and selected for continuous growth. The cells were subjected to the same schedule of passing as the NZB-Q cells and were assayed for xenotropic virus production by co-cultivation and for ecotropic virus production by XC plaque assay (Rowe et al. 1970). They were also observed at each passage for spontaneous transformation.

The NIH/3T3 cells spontaneously transformed after 11 passages but these cells did not produce any detectable virus by either the XC or co-cultivation assays. Conversely BALB/3T3 cells maintained their normal morphology, but produced small amounts of X-tropic virus.

Response of primary mouse embryo cells to repeated passage

We also investigated whether primary embryo cells would undergo spontaneous transformation after multiple cell passages. Two NZB mouse embryos were prepared and transferred as described previously (Levy, 1973). These cells are known to be spontaneous producers of xenotropic virus (Levy et al. 1975). NIH Swiss and BALB/c mouse embryo cells, and NIH mouse embryo cells infected with the AKR strain of MuLV, were passed in parallel as controls. The latter cell line assessed the influence of chronic MuLV production on the frequency of cell transformation. Assays of virus production and transformation were carried out weekly as described above.

Even after 15 passages the NZB embryo cells showed no transformation. However, both embryos studied were producing high titres of xenotropic virus (1 to $3 \times 10^6$ f.f.u./ml).
Similarly neither the BALB/c nor NIH embryo cells showed foci, even when the latter cells were producing large quantities of ecotropic MuLV. After 5 to 7 passages, the untransformed NIH Swiss mouse embryo cells began to produce very low levels of xenotropic virus. This observation contrasts with the situation found for NIH-3T3 cells, which never released detectable quantities of X-tropic virus.

**DISCUSSION**

Several studies have shown that different clones derived from the same parental mouse cell line can vary in their ability to produce ecotropic MuLV. For instance, some subclones of a cloned BALB/3T3 cell line produced virus while others did not (Lieber & Todaro, 1973). The type of virus produced may also vary among clones derived from the same parental cells (Yoshikura & Hirokawa, 1974; Hopkins & Jolicoeur 1975). Moreover, clones derived from a single mouse may vary up to 100-fold in virus production in response to iododeoxyuridine induction (Yoshikura, 1975). These observations indicate a variability in the intracellular regulation of endogenous ecotropic mouse viruses.

We have studied spontaneous production of another class of endogenous mouse type C viruses, the xenotropic viruses. All clones derived from the established NZB-Q mouse cell line produced xenotropic virus but at different titres; the level of production varied between \(10^4\) and \(10^6\) infectious particles/ml. Virus production by each clone was constant over several passages. In addition, a series of subclones derived from each clone all produced titres of virus identical to that of the parental clone.

These data indicated that our NZB-Q line was composed of cells with varying degrees of virus expression, which could be separated by cloning. The cloned cells maintain their characteristic levels of virus production over many cell divisions.

We previously demonstrated that clones of cells derived from a single NZB embryo vary in NZB-MuLV production to the same degree as the clones described here (Levy et al. 1975). These observations suggest that individual mouse cells differ in their intracellular regulation of xenotropic virus expression but that this control, once established, remains constant. Since all NZB cells contain the same number of proviral DNA sequences (Chattopadhyay et al. 1974; S. K. Chattopadhyay, personal communication) differences in genome copies cannot explain these variations. It is conceivable that certain clones are heteroploid, so that augmentation in the number of proviral genome copies may have occurred, but our observations on the variation in virus production by clones of primary embryo cells (Levy et al. 1975) argue against this possibility.

In long term culture, mouse cells often spontaneously transform and this has been ascribed to an increased expression of ecotropic type C virus genes (Lieber & Todaro, 1973). Our results with NZB and NIH Swiss cells that lack ecotropic virus expression and with cells of other mice indicate that X-tropic virus production does not correlate with transformation. The transformation of the NZB clones occurred much earlier than is usual with other mouse cells in culture, but no correlation between either the time of appearance or the number of transformed cells, and the level of virus production by any NZB clone was seen. For instance, clone 4, the lowest producer of NZB-MuLV, showed transformation at an earlier passage than clone 1, one of the highest producers. Moreover, clone 9, although producing 10-fold less NZB-MuLV than clone 35, showed 10 times more foci than clone 35. Production of virus by transformed NZB cells is also not markedly different from production by unaltered cells from the same clone. Therefore, spontaneous cellular transformation, whatever its cause, does not alter the intracellular control of virus production.

Experiments performed with NIH/3T3 and BALB/3T3 cell lines confirmed the lack of
a relationship between transformation and xenotropic virus production. NIH/3T3 cells became transformed spontaneously but were not producing any detectable infectious virus whereas BALB/3T3 cells remained normal while producing small amounts of xenotropic virus. Finally, cells from newly obtained NZB embryos were producing consistently high titres of virus and remained completely normal in appearance. We conclude, therefore, that spontaneous transformation of mouse cells is not related simply to the production of endogenous X-tropic virus.

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