Biological Characteristics of Type C Viruses Isolated from Different Friend Erythroleukaemic Cells

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SUMMARY

We have examined the expression of type C RNA viruses in different Friend erythroleukaemic cell types, distinguished on the basis of increasingly malignant characteristics, which arise during the ageing of mice inoculated with the polycythaemic Friend virus complex. Most early appearing Friend cells (type I) expressed ecotropic virus, but cells of later malignant types showed decreased and variable expression. In general, the more malignant cells released less ecotropic virus. Xenotropic virus was detected in low numbers from type I, II, and IV cells. Two viruses were cloned from type II tumour cells: a xenotropic virus (II clone 1) and an N-tropic ecotropic virus (II clone 2). No pathogenic activity was found when II clone 1 was inoculated into newborn and adult DBA/2J and NIH/Swiss mice observed for up to 20 months, whereas II clone 2 caused a rapid anaemic erythroleukaemia in both N- and B-type newborn mouse strains. It caused a similar form of leukaemia in susceptible N-type adult mice, but at a lower frequency and with a longer latency (usually >5 months). This finding demonstrated a lack of NB restriction in newborn mice. The virus was much less active in DBA/2J mice from which it had been originally cloned; it also appeared to cause lymphoma or to shorten the latency of spontaneous lymphoma in DBA/2J mice.

INTRODUCTION

Two forms of Friend murine leukaemia have been described: anaemia (FLV-A), originally reported by Friend (1957) and polycythaemia (FLV-P) which appeared after passage of the original Friend virus in HA/ICR Swiss mice (Mirand, 1966). A replication-defective spleen focus-forming virus (SFFV) (Axelrad & Steeves, 1964) and a replication-competent helper virus can be isolated from mouse tissues in both the anaemic and polycythaemic forms of the leukaemia (Friend et al., 1979; MacDonald et al., 1980a; Steeves, 1975; Troxler et al., 1980). The induction of anaemia or polycythaemia is characteristic of the virus preparation, in particular the defective viruses (MacDonald et al., 1980b; Troxler et al., 1980). While both SFFV types share RNA sequences with the ecotropic helper, they possess in addition, unique xenotropic sequences (Evans et al., 1980; Mak et al., 1978; Troxler et al., 1977, 1978).

In polycythaemic Friend leukaemia we have described four general cell types (originally termed stages) which form a progression of increasing malignancy (Levy et al., 1979). Cell type I is an erythroblast which arises in spleens of infected mice early after virus inoculation. It remains capable of differentiation and can neither form tumours nor proliferate in vitro. Cell type II, also an erythroid precursor cell, is unable to differentiate; it forms tumours at the site of subcutaneous implantation. These tumour cells grow poorly, if at all, in vitro. The cell
type III is isolated from among the subcutaneous tumour cells or from late leukaemic spleen tissue by its ability to grow in vitro. This cell is undifferentiated, tumourigenic and unresponsive to chemicals which induce erythroid differentiation, such as dimethylsulphoxide (DMSO) (Friend et al., 1971), haemin (Ross & Sautner, 1978) and hexamethylene-bisacetamide (Reuben et al., 1978). We have shown that highly tumourigenic cell type IV can arise in vitro from cloned type III cells. It may also be isolated and cloned directly from the type II tumours or from spleens of mice late after virus infection. While undifferentiated, it differs from type III cells by its increased tumourigenicity and its ability to differentiate when treated with inducers such as DMSO (Levy et al., 1979).

Studies in vivo with different strains of mice have shown that: (i) a helper virus and SFFV are both required for rapid leukaemogenicity in adult mice (MacDonald et al., 1980a; Troxler et al., 1977, 1980); (ii) the helper virus cloned from the Friend anaemia or polycythaemia complex is not pathogenic in adults but causes leukaemia in newborn mice (Oliff et al., 1980; MacDonald et al., 1980a; Troxler & Scolnick, 1978); (iii) the erythroleukaemic potential of the virus depends upon both viral and host determinants (Odaka & Yamamoto, 1962; Lilly, 1967; Suzuki & Axelrad, 1980). We present data which confirm and extend these observations.

METHODS

Mice. Newborn or 6 to 8-week-old mice were used. DBA/2J, SWR and Balb/c came from Jackson Labs, Bar Harbor, Maine; NIH/Swiss mice came from R. S. Schwartz, Tufts University Cancer Research Center.

Tissue culture. The following fibroblast cultures were used for virus assays: mink lung cells (MLC), the CCL-4 line (Henderson et al., 1974) from J. Hartley, NIH; S⁺L⁻ mink cells (Peebles, 1975) and SC-1 (Hartley & Rowe, 1975) from J. Levy, San Francisco; NIH/3T3 and Balb/c mouse embryo cells and XC rat cells (Klement et al., 1969) from R. S. Schwartz. Mink, NIH/3T3, Balb/c and SC-1 cells were maintained in McCoy's medium supplemented with 10% foetal bovine serum and gentamicin (100 µg/ml). S⁺L⁻ mink cells were passaged in RPMI medium supplemented with 10% heat-inactivated foetal bovine serum, gentamicin, 1% non-essential amino acids; polybrene (1 µg/ml) was included in assays. XC cells were grown in minimal essential medium (MEM) at 4 × concn. supplemented with 10% foetal bovine serum, gentamicin and 1% L-glutamine.

Friend cell types. Types I cells came from leukaemic DBA/2J spleens 16 days after FLV-P inoculation. Type II cells were from tumours isolated from either inoculation of leukaemic spleens subcutaneously (e.g. MT1) or by propagation of foci in spleens (SP) or omentum (OM) following inoculation of irradiated mice with 10⁶ spleen cells from aged DBA/2J leukaemic mice (method of Tambourin et al., 1980). These latter tumour cells were designated SP1, SP2, SP6 and OM1. The in vivo passage was given as TP1, TP2, etc. Type III and IV cells were cloned from subcutaneous tumours. They were maintained in suspension cultures in Eagle's basal medium (Gibco) supplemented with 10% foetal bovine serum and gentamicin. TP63 cells were cloned from a cell line originating from MT1 tumour in its 63rd passage in vivo; C7D (type IV) cells were cloned from type III cells originating from the same tumour at an earlier passage (TP46). The derivation of other type III and IV cells is given in Table 1.

Virus stocks. The FLV-P virus preparation was a 20% filtered spleen extract from 14 to 21-day-old FLV-P-infected DBA/2J mice and was originally obtained from C. Friend and passaged through DBA/2J mice for 8 years in our laboratory. The FLV-A spleen extract was obtained from C. Friend and had been passaged in NIH/Swiss mice.

Assays of viruses in vitro. Assays for virus activity were performed on mink (for xenotropic virus) and/or mouse (for ecotropic virus) fibroblast lines.
Viruses isolated from Friend leukaemic cells

Immunofluorescence tests. Fluorescent antibody assays were performed on confluent fibroblast cultures containing 22 x 22 mm sterile glass coverslips (Hartley & Rowe, 1976). After gentle rinsing with 0-01 M-PBS, they were fixed in acetone and stained with a fluorescein-labelled anti-Moloney p30-conjugated serum (obtained from J. Gruber, NIH).

S⁺L⁻ focus-forming assay for xenotropic virus. S⁺L⁻ mink cells were used as described by Levy (1977).

XC plaque assay. NIH, SC-1 and Balb/c mouse embryo cells were used to detect ecotropic virus (Rowe et al., 1970) or virus-producing cells as infectious centres (Melief et al., 1975).

Virus neutralization studies. These tests were performed as described by Levy et al. (1975) with anti-xenotropic virus sera, prepared against NZB xenotropic virus, and anti-FMR (anti-ecotropic) sera obtained from J. Levy, who performed some of these studies for us. A 67% decrease in virus titre was indicative of neutralization.

Detection of virus in Friend leukaemia cells. Cells from different populations of the four cell types were treated with mitomycin C (Kawashima et al., 1976) and inoculated at different dilutions on to DEAE-dextran-treated mink or mouse indicator fibroblast cultures (Vogt, 1967) for detection of virus activity (Melief et al., 1975). The assay was linear with cell dilution.

Cloning of xenotropic and ecotropic viruses. From the 6th cell-free passage of the filtered supernatants above MLC originally co-cultivated with MT1 cells two viruses were cloned. The xenotropic virus II clone 1 was cloned through three limiting dilutions (Hartley & Rowe, 1976) on MLC, selecting the last dilution containing activity by immunofluorescence as well as the dilution beyond this activity for each subsequent passage.

An aliquot of the same MLC supernatant which continued to exhibit XC-positive virus despite 6 cell-free passages was also used to clone the ecotropic virus (II clone 2) on SC-1 cells. The sample was diluted to give approx. 1 XC plaque per 10 cloning tissue culture wells. Forty wells were examined for XC reactivity; three were found positive with a single XC plaque. The supernatants from these were retrieved and used to propagate virus. One of these was chosen for cloning, which was done by three limiting dilutions through SC-1 cells.

Biological activity of virus in vivo. The leukaemogenicity of different virus preparations was examined in newborn and adult DBA/2J, SWR, NIH/Swiss, and Balb/c mice. Pathogenicity of the original anaemia-producing (FLV-A) and polycythaemia-producing (FLV-P) virus complexes was assayed in adult and newborn mice inoculated intraperitoneally with 0-1 ml of 2% filtered spleen extract. Newborn mice were injected intraperitoneally within 48 h of birth with 0-1 to 0-5 ml of cloned viruses, II clone 1 or II clone 2, each obtained as a filtered supernatant from tissue culture media. Adult mice received 0-1 to 0-5 ml of the cloned virus preparations.

RESULTS

Virus expression in different Friend cells

Between 10 and 30% of leukaemic, non-tumourigenic spleen cells (type I) produced XC-positive virus. Among the tumourigenic cells (types II to IV), the number of virus-producing cells was variable (Table 1) even within phenotypically characterized cell types. Low virus expression was characteristic of the more malignant cell types. Moreover, as cells were passaged in vivo and in vitro, virus production appeared to decrease further (Table 1). Type III cell clones from one tumour passage, e.g. TP63, were an exception; virtually all cells expressed ecotropic virus.

When co-cultivated with MLC, none of several representative cell types initially showed detectable xenotropic virus by immunofluorescence or foci on S⁺L⁻ cells. However, after 1 or
Table 1. Ecotropic virus expression in different Friend cells

<table>
<thead>
<tr>
<th>Cells tested</th>
<th>Malignant cell type*</th>
<th>No. infectious centres per 10^6 cells†</th>
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<tbody>
<tr>
<td>Leukaemic spleen cells‡</td>
<td>I</td>
<td>1·8 ± 1·7 × 10^5</td>
</tr>
<tr>
<td>Subcutaneous tumours‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT1 TP110,115</td>
<td>II</td>
<td>2·2 ± 0·6 × 10^3</td>
</tr>
<tr>
<td>MT1 TP204,205</td>
<td>II</td>
<td>4·5 ± 2·1 × 10^3</td>
</tr>
<tr>
<td>SP1 TP16</td>
<td>II</td>
<td>1 × 10^3</td>
</tr>
<tr>
<td>SP2 TP16</td>
<td>II</td>
<td>4 × 10^3</td>
</tr>
<tr>
<td>SP6 TP14</td>
<td>II</td>
<td>3·8 ± 2·3 × 10^1</td>
</tr>
<tr>
<td>OM1 TP13</td>
<td>III–IV</td>
<td>4·1 ± 1·9 × 10^3</td>
</tr>
<tr>
<td>Cloned cell lines in vitro from MT1 tumour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP46-C7-C7Dp208,221</td>
<td>IV</td>
<td>2·8 ± 3·4 × 10^2</td>
</tr>
<tr>
<td>TP46-C7-C7Dp504,510</td>
<td>IV</td>
<td>3 ± 1·4 × 10^3</td>
</tr>
<tr>
<td>TP63-D4p57</td>
<td>III</td>
<td>1 × 10^6</td>
</tr>
<tr>
<td>TP63-D6p63</td>
<td>III</td>
<td>&gt; 10^3</td>
</tr>
<tr>
<td>TP110-A6p204</td>
<td>III</td>
<td>5 × 10^3</td>
</tr>
<tr>
<td>Cloned cell lines in vitro from SP1 tumour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-B6p24</td>
<td>III</td>
<td>8 × 10^2</td>
</tr>
<tr>
<td>Cloned cell lines in vitro from SP2 tumour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-A2p3</td>
<td>III</td>
<td>&lt; 10^3</td>
</tr>
<tr>
<td>B-B4-4p8</td>
<td>IV</td>
<td>&lt; 10^3</td>
</tr>
<tr>
<td>B-B4-5p5</td>
<td>III</td>
<td>&lt; 10^3</td>
</tr>
</tbody>
</table>

* As determined by biological activity in vivo, DMSO responsiveness and by ratio of H2a variants (Levy et al. 1979).
† Tested on NIH/3T3 cells.
‡ No difference with or without lysis of red cells by ammonium chloride treatment.

2 passages of the MLC originally cultivated with one representative cell population of each of the four cell types, only in I, II and IV, was xenotropic virus detected. Approximately 25 to 40% of these passaged MLC showed immunofluorescence. No xenotropic virus was detected above the MLC co-cultivated with TP63-D4 for 9 passages. The virus from above the MLC was tested for ability to replicate in mouse cells. A less than twofold ecotropism was found. The possibility that the activity represented dual-tropic viruses or were the result of phenotypic mixing required cloning.

**Characterization of cloned xenotropic and ecotropic virus from type II cells**

The cloned II clone 1 proved to be a pure xenotropic virus by assays on mouse and mink cells; II clone 2 showed only ecotropism. It was N-tropic by virtue of about 10^3 higher titre of XC positivity on NIH/3T3 cells (10^6.0 plaques/ml) versus Balb/c (10^3.3 plaques/ml). The tropism resembled the XC-positive virus in the original FLV-P complex which titred at 10^6.7 plaques/ml in NIH/3T3 and 10^3.8 plaques/ml in Balb/c. II clone 1 was neutralized by anti-xenotropic sera, but not anti-ecotropic; II clone 2 was neutralized by anti-ecotropic sera, but not anti-xenotropic sera (data not shown).

**Biological activity in vivo of cloned viruses**

The activity of the cloned viruses in vivo was tested in four mouse strains. For comparison, we also examined the activity of the uncloned FLV-P and FLV-A preparations. The original FLV-P complex produced splenomegaly and polycythaemia (haematocrit > 50%) in NIH/Swiss, DBA/2J newborns and adults in 2 to 3 weeks. It was not leukaemogenic in adult Balb/c mice observed for more than 7 months. An NB-tropic FLV-A complex produced splenomegaly and anaemia (haematocrit < 30%) in NIH/Swiss, DBA/2J and Balb/c mice in 2 to 4 weeks.
II clone 1 caused no pathological symptoms in either of two mouse strains tested: 26 newborn and 11 adult DBA/2J, and 11 newborn and 6 adult NIH/Swiss were inoculated with 0.1 ml (10^5 fluorescent loci) intraperitoneally; 5 adult DBA/2J were inoculated intravenously. Many animals were observed for over 20 months, with bi-weekly observations of growth and development, size of spleen, and peripheral blood haematocrit. Newborn mice inoculated with II clone 1 showed slightly lower weights at 2 to 5 weeks, but they were similar to controls by 6 to 8 weeks. In contrast, inoculation with II clone 2 caused splenomegaly and anaemia in newborn mice from four strains, and a similar but less frequently resulting disease in adults. The rapidity of onset of the disease was also noticeably different (Table 2).

Of the N-type mouse strains tested with the virus, SWR was the most susceptible. Newborns injected with 0.1 ml (10^5 XC-positive virus) developed disease very rapidly, some within 2.5 months (Table 2) with haematocrits of 20 to 21%. Newborn NIH/Swiss mice rapidly developed disease (most within 4 months), as characterized by splenomegaly and anaemia. Mice injected with a larger virus inoculum (0.3 to 0.5 ml) developed the disease more rapidly (1.5 to 3.5 months) and with more severe anaemia. Adult Swiss mice were less susceptible at the lower virus inoculum and slower to develop this disease. More mice became leukaemic with a threefold larger virus dose, but the latency was still 7 to 8 months.

II clone 2 had notably low leukaemogenic activity in DBA/2J mice from which it was obtained originally: no effect on newborns or adults was detected for at least 4 months. Of the mice inoculated with 0.1 ml of II clone 2, 18% of the newborns and 30% of the adults developed anaemia and splenomegaly after more than 6 months (Table 2). Larger doses (0.3 ml) of virus did not produce a more severe or frequent disease in adults (in contrast to results with NIH/Swiss) but appeared more active in newborns. A small percentage of the mice (7% of the newborns; 4% of the adults) developed lymphoma with enlarged spleens, lymph nodes and/or thymus within 5 to 10 months.
Newborn Balb/c mice were susceptible to II clone 2. They developed splenomegaly and anaemia in 3 to 4 months with a mean haematocrit of 25% (Table 2). The incidence was similar to NIH/Swiss but lower than SWR. This result demonstrated that NB tropism was not restricting leukaemogenesis in newborns.

There was no evidence of contaminating SFFV-A or SFFV-P in these stocks which were tested after they had been extensively passed in fibroblast cultures. Assays were negative in vitro as well as in vitro. The latter consisted of (i) oligonucleotide mapping which showed only helper virus in the ecotropic virus preparation (C. Thomas, J. Coffin & S. Levy, unpublished results) and (ii) testing of virus on bone marrow cells which showed no erythroid burst activity in either virus preparation tested alone or tested when mixed together (D. Hankins, personal communication; Hankins & Troxler, 1980).

**DISCUSSION**

Virus expression did not correlate directly with the progression of the malignant process from the in vivo, non-tumourigenic type I cell to the in vitro, highly malignant type IV cell. However, there does appear to be decreased virus production with increased malignancy. The findings suggest that in different cell types, the virus gene(s) is organized differently in the total cell chromatin. The varied levels of expression of these viruses may reflect the different chromatin structure described in these cell types (Leonardson & Levy, 1980).

Because cell type II represented a dramatic change in malignant potential from the non-tumourigenic, differentiating type I cell, we decided to clone and characterize the ecotropic and xenotropic viruses from the 6th supernatant passage of MLC originally co-cultivated with MT1 cells to determine if any recombinant viruses were present and if biological activities were unique to these viruses. The XC-positive virus, noted originally, remained consistently present despite 6 cell-free passages on MLC.

Two cloned viruses were isolated from type II cells. II clone 1 is presumably an endogenous xenotropic virus maintained by the DBA/2J. II clone 2 which is an ecotropic leukaemogenic virus, was unusual in its propagation prior to cloning in serially passaged supernatants on mink cells which should have selected against ecotropic virus. The mechanism for maintenance of II clone 2 through MLC must have resulted from phenotypic mixing (Levy, 1977). While we detected no dual-tropic viruses (Troxler et al., 1978), their presence in lower titre cannot be excluded. SFFV of either A or P type was not demonstrated in stocks of this virus by tests in vivo and in vitro.

Inoculated mice have been followed for over 1.5 years to document the short- and long-term biological effects. To date, the xenotropic virus (II clone 1) has produced no leukaemia or altered growth or blood changes in newborn or adult mice observed for up to 20 months. As with other endogenous xenotropic viruses, the in vivo function of II clone 1 is unclear.

In contrast, II clone 2 virus is a replication-competent ecotropic virus. Isolated from mice inoculated with the polycythaemic virus complex, it caused a rapid anaemic form of erythroleukaemia in newborn SWR mice, a slower appearing anaemic erythroleukaemia in NIH/Swiss newborns and adults, and in Balb/c newborns, and a much longer onset and lower frequency of anaemic erythroleukaemia in a few DBA/2J newborns and adults (Table 2). The induction of an anaemic form of erythroleukaemia in newborns by II clone 2 resembles the activity of helper virus isolated from other Friend virus complex preparations (Troxler & Scolnick, 1978; MacDonald et al., 1980a). However, there are differences. The other helper viruses are NB-tropic in vitro and show equal leukaemogenicity in N- and B-type newborn mice. II clone 2, while equally leukaemogenic in N- and B-type mice, tests N-tropic in vitro. The FLV-P from which II clone 2 was cloned was non-leukaemogenic in
Viruses isolated from Friend leukaemic cells

Balb/c. Thus, it appears that NB-tropism does not affect the erythroleukaemic potential of this cloned virus in vivo.

Moreover, among N-type mice, II clone 2 revealed different susceptibilities. In particular, it was much less virulent in DBA/2J mice, the strain from which it had been originally isolated. These differences among N-type strains suggest that genes other than Fv-1 and Fv-2 are involved in the biological activity of the cloned Friend helper virus. II clone 2 appeared to show greater activity in adult mice than reported for the other helper viruses described above. However, these mice were generally followed for less than 2 months which may not have been long enough to see the disease. Since non-defective helper viruses from the anaemia and polycythaemia virus complexes both cause anaemia, the differences in the leukaemias caused by the anaemia versus polycythaemia complex must relate to the replication-defective virus, i.e. SFFV, as has been discussed by others (MacDonald et al., 1980 b; Troxler et al., 1980).

In addition to the erythroleukaemic potential of II clone 2, the virus appeared to enhance or induce a thymic lymphoma in some DBA/2J mice. Spontaneous lymphoma develops in DBA/2J at a rate of 10% in 81 weeks (Smith et al., 1973). Thus, the lymphoma-producing potential of II clone 2 may be in doubt. However, our data suggest that the lymphomas observed in our studies resulted from the inoculation of II clone 2, since a similar follow-up of DBA/2J mice injected with II clone 1 showed no lymphoma. Moreover, the lymphomas developed 10 to 15 months earlier than has been reported for spontaneous disease in DBA/2J. Dawson et al. (1966) reported lymphatic leukaemia following inoculation of Balb/c mice with a replication-competent virus recovered from rats inoculated with Friend complex. Since only adults and one strain were examined, it is possible that these viruses were also erythroleukaemogenic in newborns. Others have demonstrated a lymphatic leukaemia virus in Friend virus extracts (for review, see Gross, 1970).

While non-Friend type C RNA viruses can complement SFFV-P or SFFV-A causing the respective leukaemia, there remains the question whether the cloned Friend helper with its innate leukaemia-inducing properties enhances malignant potential. We do not know, for instance, what role the virus (or other viruses) plays in the transition from the differentiating type I cell to the tumourigenic type II cell. Is a leukaemogenic helper required? Are tumour cells demonstrable in mice inoculated with cloned helper virus? These questions are under study. Biochemical and biological studies of our cloned viruses and their comparison with other helper viruses may help to clarify the relationship of these viruses to the progressive malignant nature of this leukaemia.

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Viruses isolated from Friend leukaemic cells


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