An IgM-producing B Lymphoblastoid Cell Line Established from Lymphomas Induced by a Non-defective Reticuloendotheliosis Virus


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SUMMARY

Chick syncytial virus (CSV), a strain of avian reticuloendotheliosis virus (REV) causes lymphoid tumours in chickens after a prolonged incubation period. A number of CSV-induced tumours were examined for cell surface antigen and were found to be of the B cell type and to produce immunoglobulin. Attempts were made to grow in vitro cell lines from CSV-induced tumours and a lymphoblastoid cell line was established from a liver tumour of a chicken that was inoculated with CSV via the yolk sac in embryo. The donor chicken was viraemic at the time the tumour was removed. The cell line is designated RECC-RP13, it produces non-defective REV, is a B cell type and it produces IgM. It is free from infection with endogenous and exogenous avian leucosis virus (ALV) and has an increased number of chromosomes. Sequences specific to REV were detected in at least four sites in cellular DNA from RECC-RP13. Sequences specific to ALV DNA, beyond that normally found in 15I_5 x 7_1 cells, were not found in DNA from this cell line.

INTRODUCTION

Viruses belonging to the reticuloendotheliosis (REV) group are (Purchase et al., 1973) avian oncogenic retroviruses. They can be distinguished from avian leucosis viruses (ALV) on the basis of their morphology (Zeigel et al., 1966), reverse transcriptase (Mizutani & Temin, 1973), envelope glycoproteins (Theilen et al., 1966), group-specific antigens (Maldonado & Bose, 1973) and RNA base sequences (Kang & Temin, 1973). Furthermore, REV appears to be related to the mouse leukaemia viruses on the basis of its reverse transcriptase (Moelling et al., 1975).

REV has been isolated from turkeys (Robinson & Twiehaus, 1974), ducks (Ludford et al., 1972) and chickens (Cook, 1969). Strain T prototype REV, which was originally isolated from turkey tumours after repeated, rapid bird passage, consists of a replication-defective, highly oncogenic virus, and a non-defective helper virus that causes immunosuppression in the chicken (Hoeltzer et al., 1979). The non-defective helper REV (ndREV) replicates well in chicken embryo fibroblast cultures (CEF) but fails to transform lymphoid cells in vitro. Conversely, the replication-defective REV (rdREV) fails to replicate in CEF cultures but transforms lymphoid cells in vitro. Also, rdREV causes an acute neoplastic response in chickens, whereas the ndREV causes an immunosuppression associated with running and poor growth. Recently, Witter & Crittenden (1979) showed that chickens inoculated with chick syncytial virus (CSV), an ndREV, develop lymphomas at about 17 to 35 weeks post-inoculation; Grimes et al. (1979) observed a similar result. A striking similarity also
exists between the lymphomas induced by ndREV and those induced by ALV, which are discussed by Witter & Crittenden (1979) and Witter et al. (1981).

In this report we demonstrate the B cell characteristic of CSV-induced lymphomas, describe the establishment of a lymphoblastoid cell line from a CSV-induced tumour and show that the cell line is also of the B cell type and produces IgM. We further demonstrate that this cell line is free from expression of endogenous and exogenous ALV and that CSV is the most likely cause of its transformation.

METHODS

Chickens. F1 progeny of line 15I5 males and line 71 females maintained at the Regional Poultry Research Laboratory in East Lansing, Michigan were used. Parent chickens were free from infection with REV, exogenous ALV and Marek's disease virus (MDV). Chickens of 15I5 × 71 progeny are expected to produce endogenous ALV. Line 72 chickens were used as a source of spleen cells for in vitro transformation.

Cells. CEF cultures were used for virus assay. The CEF cultures were grown and maintained in medium 199 and F10 mixture supplemented with 2 to 4% calf serum. The RECC-UT1 lymphoblastoid cell line transformed by strain T, an rdREV, was kindly provided by Dr H. Bose. This was grown in RPMI 1640 medium supplemented with 10% foetal bovine serum and will be referred to as the UT1 cell line.

Viruses. CSV, a strain of ndREV, was used for inoculation of chickens. The CSV (Cook, 1969) was propagated in CEF cultures and was previously clone-purified. Rous-associated virus RAV-1 (a subgroup A exogenous ALV), RAV-0 (an endogenous ALV) and CSV were used for inoculation of cell cultures.

Antibodies. All antibodies were raised in rabbits. Appropriate chicken cells were used for absorption of non-specific reactivity. Antisera to chicken B cell and chicken T cell are those previously described (Sharma, 1977). Antisera to chicken IgM and IgG were kindly provided by Dr E. J. Smith. Antisera to purified group-specific antigen of ALV and to purified REV were those previously described (Smith, 1977; Smith et al., 1977).

Complement fixation. CEF cultures were infected with the appropriate viruses and a 20% extract of infected cultures was used as a source of antigen in complement fixation tests for ALV (COFAL) and complement fixation for REV (COFAR) tests as described previously (Smith, 1977; Smith et al., 1977).

Membrane immunofluorescence. Lymphoma cells or lymphoblastoid cells in suspension were washed twice in phosphate-buffered saline (PBS) and then incubated with 1:10 dilution of appropriate sera for 30 min at 4 °C. Cells were then washed twice in PBS and incubated in fluorescent conjugated anti-rabbit immunoglobulin for 30 min at 4 °C. Cells were then washed twice in PBS, mounted on glass slides and examined with a Leitz u.v. microscope equipped with a vertical illuminator.

Chromosome analysis. Samples for analysis of chromosomes were prepared as previously described (Shaffner et al., 1967). Colcemid was added to lymphoblastoid cell cultures to reach a concentration of 3 mg/ml. Cultures were then incubated at 37 °C for 2 h. Cells were removed by centrifugation, resuspended in 0-062 M-KCl and fixed in three changes of 1:3 methanol:acetic acid. Cells were then placed on clean glass slides, stained with carbol-fuchsin and examined with a Leitz microscope.

Electron microscopy. Tumour tissues and pelleted lymphoblastoid cells were fixed in 1% OsO4 and prepared for electron microscopy as previously described (Nazerian & Burmester, 1968). Thin sections were stained with uranyl acetate and lead citrate and examined with a Siemens Elmiskop 1A electron microscope.

DNA analysis. The extraction of DNA from cultured cells, agarose gel analysis and Southern-filter hybridizations all follow previously described procedures (Fung et al., 1981).
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Briefly, cells were lysed by SDS-Pronase treatment and the high mol. wt. chromosomal DNAs were purified through phenol-chloroform extraction. The DNA samples were then subjected to digestion with EcoRI or SacI (New England Biolabs, Beverly, Mass., U.S.A.) endonucleases. The resulting DNA fragments were electrophoresed on a 0.8% agarose gel and transferred on to a nitrocellulose filter paper (Millipore) by the method of Southern (1975). The DNA immobilized on filter paper was then hybridized with the following DNA probes. (i) For detection of CSV DNA sequences, a cloned spleen necrosis virus (SNV) DNA (kindly provided by Dr H. Temin), carrying the entire genomic sequences of SNV (a strain of REV), was nick-translated by the method of Maniatis et al. (1975) to yield DNA probes with high radioactivity. (ii) For detection of ALV or ALV-related endogenous virus (ev) DNA sequences, radioactive complementary DNA (cDNA) was prepared using the 70S RNA template and the endogenous reverse transcriptase present in the detergent-activated virions of ALV (Fung et al., 1981). Both DNA probes were labelled with $^{32}$PdNTP (New England Nuclear) to a specific activity of $10^8$ ct/min/μg. Autoradiography of the filter was carried out at −70 °C using Kodak XR-omat film.

RESULTS

Development of the cell line

Lymphoid tumours of progeny chicks from 15I 5 males and 7 I females, initially inoculated with CSV in the embryo or at 1-day-old, were aseptically removed and made into a cell suspension. Most of the donor birds had multiple tumours and often tumours in the bursa, but there were a few birds that lacked bursa tumours. Cell suspensions were purified on Ficoll-Hypaque gradients and were grown in RPMI 1640 medium supplemented with 20% foetal bovine serum and 10% tryptose phosphate broth. Cultures were incubated at 37 °C in 5% CO₂. Only one cell line was established from 22 CSV-induced tumours used. The cell line is designated RECC-RP13 according to the nomenclature of Witter & Crittenden (1979) and will be referred to as RP13 in this paper. It has been in culture for 18 months and serially passaged 200 times. The doubling time of an RP13 culture is 8 to 12 h. Cultures were routinely passaged every 48 h. RP13 cells measure 8 to 10 μm in diam., are lymphoblastoid in morphology and grow as single-cell or as small cell aggregates. A considerable number of RP13 cells have an increased number of chromosomes often near or beyond tetraploidy (Fig. 1). However, normal diploid cells are also present (Fig. 2). Besides an increase in the number of chromosomes, no obvious chromosomal abnormality is observed (Fig. 3). Electron microscopic examination of the original tumour from which the RP13 cell line was established, as well as other CSV-induced tumours, showed a number of large lymphoid cells with a smooth surface (Fig. 4). Cells grown in culture (RP13), however, have lymphoblastoid morphology but a rough surface (Fig. 5). These cells produce complete virus particles with typical REV morphology (Fig. 5, inset).

Surface antigenic markers on CSV-induced tumours and RP13 cells

Bursa, liver and gonad tumours from seven chickens inoculated with CSV were removed and prepared in single-cell suspension and were indirectly stained with sera raised in rabbits against chicken B cells, chicken T cells and chicken IgM and IgG. RP13 cells were stained similarly. The results are given in Table 1. All tumours stained positively against anti-B cell serum (9/9) but failed to react with anti-T cell serum. A number of tumours were also positive against anti-IgM serum (5/7) but all were negative against anti-IgG serum. Thus, all tumours examined were of B cell type and the majority of these tumours also produced IgM. Similar observations were made with the RP13 cell line: these cells stained positively with anti-B cell
Fig. 1. Chromosome spread of an RECC-RP13 cell. Approximately 40 to 50% of cells like this one have an increased number of chromosomes.

Fig. 2. A chromosome spread of an RECC-RP13 cell with the normal number of chromosomes.

Fig. 3. Karyogram of a cell with more than the normal number of chromosomes. This particular cell appears to be tetraploid but certain chromosomes like numbers 1, 3, W, 9 and 15 are more than tetraploid.

Fig. 4. Electron micrograph of cell suspension from a CSV-induced liver tumour. Tumour cells have the appearance of lymphoid cells and have a smooth surface. These cells produce virus particles with REV morphology. Bar marker represents 2.5 μm.

Fig. 5. (a) Electron micrograph of cell from RP13 cell line. Although, morphologically, these cells have a lymphoblastoid appearance their rough surface projections make them distinguishable from the tumour cells. Bar marker represents 2.5 μm. Insets (b to e) show the process of virus maturation and budding from the plasma membrane. All bar markers represent 6 μm.
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Table 1. Membrane immunofluorescence of CSV-induced tumour cells and RECC-RP13 cells against antisera to chicken T cells, B cells, IgM and IgG*

<table>
<thead>
<tr>
<th>Source of CSV tumour</th>
<th>B cells</th>
<th>T cells</th>
<th>IgM</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>++ (95%)</td>
<td>--</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Bursa</td>
<td>++ (95%)</td>
<td>--</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Liver</td>
<td>++ (92%)</td>
<td>--</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Bursa</td>
<td>++ (97%)</td>
<td>--</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Liver</td>
<td>NT</td>
<td>NT</td>
<td>+ (60%)</td>
<td>--</td>
</tr>
<tr>
<td>Bursa</td>
<td>NT</td>
<td>NT</td>
<td>+ (65%)</td>
<td>--</td>
</tr>
<tr>
<td>Bursa</td>
<td>+ (92%)</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Ovary</td>
<td>+ (90%)</td>
<td>--</td>
<td>±</td>
<td>--</td>
</tr>
<tr>
<td>Liver</td>
<td>++ (95%)</td>
<td>--</td>
<td>+ (48%)</td>
<td>--</td>
</tr>
<tr>
<td>Liver</td>
<td>++ (98%)</td>
<td>--</td>
<td>+ (40%)</td>
<td>--</td>
</tr>
<tr>
<td>Ovary</td>
<td>+ (95%)</td>
<td>--</td>
<td>+ (65%)</td>
<td>--</td>
</tr>
<tr>
<td>RECC-RP13 cell line</td>
<td>+ (98%)</td>
<td>--</td>
<td>+ (97%)</td>
<td>--</td>
</tr>
<tr>
<td>Normal thymus</td>
<td>--</td>
<td>++ (&gt;90%)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Normal bursa</td>
<td>++ (&gt;95%)</td>
<td>--</td>
<td>+ (7%)</td>
<td>±</td>
</tr>
</tbody>
</table>

* Intensity of membrane immunofluorescence was scored as ++, very bright; +, bright; ±, some faint reaction; --, no reaction; NT, not tested.

Table 2. Assay for infectious virus released by RECC-RP13 cell line

<table>
<thead>
<tr>
<th>Source of virus</th>
<th>Genotype of CEF-infected</th>
<th>C/E*</th>
<th>C/C†</th>
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</thead>
<tbody>
<tr>
<td>RECC-RP13</td>
<td>COFAL‡</td>
<td>COFAR§</td>
<td>COFAL</td>
</tr>
<tr>
<td>RAV-0</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>RAV-1</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>CSV</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* CEF cultures resistant to ALV subgroup E.
† CEF cultures resistant to ALV subgroup C.
‡ COFAL, Complement fixation for avian leukosis virus.
§ COFAR, Complement fixation for reticuloendotheliosis virus.

CEF cultures resistant to ALV subgroup E (C/E) and resistant to ALV subgroup C (C/C) were infected with 0.1 ml supernatant fluid of RP13 cultures. Separate cultures were also infected with RAV-0, RAV-1 and CSV. Two weeks later the cells were harvested and assayed for antigens specific to ALV or REV by COFAL and COFAR tests respectively. The results in Table 2 show that RP13 cultures produced REV as indicated by production of antigens specific to REV in both C/E and C/C cultures. On the other hand, neither C/E nor C/C cultures infected with supernatant fluids from the RP13 cultures produced GS antigens specific to ALV. As expected, C/E cells infected with RAV-1 produced ALV-specific GS antigen and C/C cells infected with either RAV-0 or RAV-1 were found positive for GS antigens specific for ALV, and both cultures were positive by the COFAR test when infected with CSV. These results indicate that RP13 cells produce REV but not endogenous or exogenous ALV.
Lymphoid cells from spleen of line 7 chickens were infected with viruses from either RP13 or UT1 (Lewis et al., 1981) cell lines. Cultures were examined for transformation and immortalization of cells. Transformation of lymphoid cells was easily observed in cultures infected with virus released by the UT1 cell line, a cell line that produces both rdREV and helper ndREV. Virus released by the RP13 cell line, on the other hand, failed to transform lymphoid cells.

**DNA analysis**

An essential step in the transformation of cells by RNA tumour viruses is the integration of virus DNA into the host cell DNA (Bishop, 1978). As a result, the tumour cell acquires specifically integrated viral DNA in the chromosome. When digested with appropriate enzymes and analysed on agarose gel, these integrated virus sequences appear as distinct bands, which are unique to the tumour cell and are not present in uninfected cells (Neiman et al., 1980; Neel et al., 1981; Payne et al., 1981; Fung et al., 1981). A previous study of 20 ALV-induced tumours (Fung et al., 1981) and 16 CSV-induced tumours (Noori-Dalloii et al., 1981) showed that in every case tumour-specific bands corresponding to the infecting virus genome were found. Thus, these tumour-specific bands were characteristic markers for the infecting virus and provided means for identification of the transforming agent. We employed this procedure to show that RP13 originated from tumours induced by CSV and not by ALV. Chromosomal DNA extracted from RP13 cells was digested with EcoRI, an enzyme which does not cleave CSV DNA (Fig. 6a). After EcoRI digestion, each newly integrated CSV DNA is contained in a single fragment, the size of which is determined by the nearest cleavage sites on the adjacent cellular sequences. As shown in Fig. 6 (b, lane 2) five bands bearing CSV sequences were detected in the RP13 chromosome. The uppermost one was also detected in the uninfected 1515 × 71 bursa cells (lane 1) and, presumably, represents the previously described endogenous CSV sequences in bursa cells of this genetic cross (Noori-Dalloii et al., 1981). The other four bands (indicated by ●) probably represent the newly acquired proviruses. The sizes of these DNA fragments are all larger than the virus genome (about 8 kb), indicating that it is linked to cellular sequences in each band. It, thus, appears that the RP13 cells carry at least four CSV proviruses integrated at different sites of the chromosomes.

Using similar methodology, we also examined the presence of additional ALV sequences in the chromosomes of RP13 cells. In this case, SacI enzyme, which has a single cleavage site near the left end of ALV DNA, was used for digestion of RP13 cell DNA (Fig. 6c). The hybridization probe was a cloned DNA carrying the entire ALV genome. A DNA sample from uninfected bursa cells from 1515 × 71 chickens (Fig. 6d, lane 1) showed four specific bands that represent four ALV-related ev loci. The SacI-digested RP13 DNA (lane 2) displayed a virus DNA pattern identical to the uninfected control; additional ALV proviruses were not seen. Digestion of the RP13 DNA samples with two other enzymes also failed to distinguish RP13 from uninfected 1515 × 71 cell DNA (data not shown). These data, taken together, suggest that the RP13 cell line has not been infected by an exogenous or endogenous ALV, and support the contention that transformation of RP13 was induced by CSV.

**DISCUSSION**

Reticuloendotheliosis viruses are known to include a single strain (T) of rdREV transforming virus that causes acute neoplastic response in the chicken and transforms avian lymphoid cells in vitro with high efficiency (Hoeltzer et al., 1979). They also include several strains or isolates of ndREV helper viruses that cause immuno-suppression and poor growth in young chickens (Hoeltzer et al., 1979), and induce lymphomas after a prolonged incubation period (Witter & Crittenden, 1979) but fail to transform lymphoid cells in vitro. Compared to
the DNA of ndREV the DNA of rdREV has a large deletion but has an additional segment known as the rel region (Chen et al., 1981) which is related to the transforming sequences of the virus and has a counterpart in normal cellular DNA (Wang & Lai, 1981). The helper virus which lacks this region is, nonetheless, capable of transforming lymphoid cells in vivo and causes frank lymphomas.

Lymphomas caused by ndREV, such as CSV, are quite similar to those produced by ALV. Witter & Crittenden (1979) showed that chickens inoculated with CSV developed lymphomas after a prolonged incubation period which were histologically very similar to those caused by ALV. They also noted that two CSV-induced tumours produced IgM. Recently, A. M. Fadly (personal communication) has observed that CSV-induced lymphoma is a B cell-dependent lymphoma since, like lymphoid leukosis, it can be greatly reduced by chemical bursectomy. In this report we have examined surface antigens of CSV-induced tumour cells from a number of chickens and the RP13 cell line that was developed from one such tumour. We found that all tumours examined and RP13 cells had surface antigenic markers similar to normal chicken B cells, and that RP13 cells and the majority of CSV-induced tumours also produced IgM. These findings, along with the previous observations cited above, show a striking similarity between

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Fig. 6. The analysis of the CSV- and ALV-related sequences in the genome of RP13 cells. (a) EcoRI cleavage map of a CSV provirus. (b) EcoRI-digested DNA from the bursa cells of an uninoculated chicken (lane 1) and from RP13 cells (lane 2). The DNA samples were analysed on 0.8% agarose gel and transferred to a nitrocellulose paper. Radioactive SNV cDNA (see Methods) was used to detect the CSV proviral DNA sequences. • indicates the newly integrated CSV DNA in RP13 cells. EcoRI-digested phage DNA was electrophoresed in parallel as molecular size markers, the positions of which are indicated on the side in kilobases (kb). (•, See text, probably represent newly acquired proviruses.) (c) The SacI cleavage map of an ALV provirus. (d) SacI-digested DNA from the bursa cells of an uninoculated chicken (lane 1) and from RP13 cells (lane 2); the hybridization probe was ALV cDNA which detects the ev sequences.
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tumours induced by CSV and ALV, and raise the possible involvement of ALV in tumours induced by CSV.

In order to show that CSV is the transforming agent of the RP13 cell line we made attempts to exclude the possible involvement of ALV in transformation. The cell line was free from infection with either endogenous or exogenous ALV. No additional bands corresponding to exogenous ALV could be found in DNA from RP13 cells, whereas at least four proviruses specific to REV were found to be newly acquired and integrated into RP13 cell DNA. These data strongly support the role of CSV in transformation of RP13 cells.

In view of the genetic unrelatedness of CSV and ALV, and the lack of oncogenes in both viral genomes, the finding that both viruses induce very similar (if not identical) neoplasm is intriguing. It suggests that the oncogene involved in such a disease is perhaps of cellular origin. This view has recently gained strong support: Hayward et al. (1981) reported that in over 90% of the ALV-induced lymphoma tumours, the expression of a cellular gene (c-myc) is elevated 10- to a 1000-fold. This cellular gene is related to the oncogene of a replication-defective myelocytomatosis virus, MC-29 (c-myc: cellular sequences homologous to the oncogene of myelocytomatosis virus). They further showed that the activation of c-myc is due to the integration of ALV provirus next to this gene (thereby promoting its expression).

We (Fung et al., 1981) and others (Payne et al., 1981; P. Neiman, personal communication) have confirmed these findings. Recently, we have further extended this study to CSV-induced lymphomas (Noori-Dalloli et al., 1981). In the analysis of 16 CSV-induced tumours, we found that the CSV provirus is integrated next to the c-myc gene in every single case. The strong correlation of lymphoid leukemia tumours and the specific integration of either ALV or CSV near the c-myc gene suggest that c-myc is a prime candidate for the oncogene involved in lymphomagenesis. It also indicates that both CSV and ALV exert their oncogenicity by inducing the expression of a cellular oncogene. At present, we do not know which of the four proviruses in RP13 cell is linked to the c-myc gene, nor do we know whether all four proviruses are contained in a single, cloned cell population. The non-uniformity of the intensity of the four proviruses (Fig. 6b) would argue for the possibility that more than one population of cells exist. On the other hand, the lower intensity of certain bands can be attributed to the deletion of a portion of the virus sequences during tumourigenesis – a phenomena previously observed by us in ALV-induced tumours (Fung et al., 1981). A third possibility is that these proviruses, represented as higher intensity bands, are situated on the amplified chromosomes. Experiments are in progress to clone the proviral DNA sequences for further structural analysis. Attempts are also being made to subclone the RP13 cell line.

The successful establishment of a CSV-induced lymphoblastoid cell line permits us to conduct a detailed, comparative study on the expression of proteins and mRNAs with several previously established cell lines (e.g. RP9, RP12) derived from ALV-induced tumours. The results obtained from such studies should yield important information concerning the common mechanism involved in transformation of B lymphocytes.

REFERENCES

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