Isolation and Characterization of Reticuloendotheliosis Virus Transformed Bone Marrow Cells

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Summary. Transformed cells have been isolated from the bone marrow of reticuloendotheliosis virus (REV)-infected, moribund chicks. These cells have been maintained in vitro through more than 30 serial passages. The cells induce solid tumors when inoculated into the wing web of day-old chickens, and cell-free filtrates induce reticuloendotheliosis. Virus particles recovered after cocultivation of bone marrow cells and chicken embryo cells band at a density of 1.16 g/cm³ in sucrose gradients. The virions contain 64S and 4S RNA species and a polypeptide composition similar to that of REV and distinct from that of Rous sarcoma virus. Complement fixation tests indicate that viruses of the avian leukosis-sarcoma complex are not present in virus concentrated from these cultures.

Reticuloendotheliosis virus (REV) (strain T) is an avian C-type RNA virus whose cardinal characteristic is its remarkable virulence [1]. REV is morphologically similar to other C-type avian tumor viruses (ATV) [2, 3]. The virions contain single-stranded 64S RNA which dissociates into 35S subunits upon denaturation and 4S RNA species [4,5]. DNA polymerase activity is associated with purified virions [6–8].

REV is, however, distinct from members of the ATV complex by several criteria [1–5,8,9]. It is antigenically distinct from the ATV [5,9, 10]. It fails to mix phenotypically with these viruses and does not induce RAV-60 or complement temperature-sensitive mutants of Rous sarcoma virus (RSV) [5]. REV also differs from ATV in its buoyant density [11]. Hybridization studies do not demonstrate a significant degree of homology between the RNAs of REV and ATV [8]. These observations indicate that REV represents a new type of avian...
RNA tumor virus. Other viruses have now been classified with REV into a new group [9].

REV replicates in chicken embryo (CE) cells but fails to transform these cells in vitro [10, 12]. Transformed cells are present in livers and spleens of REV-infected chicks [13], but efforts to culture these cells have not been successful. We have isolated cells from the bone marrow of REV moribund chicks which have characteristics of transformed cells. These cells induce tumors at the site of inoculation in day-old chickens and can be subcultured through at least 30 generations in vitro.

Materials and Methods

Virus, cells, and experimental animals. REV (strain T) and the Bryan strain of RSV were obtained from A. S. LEVINE (Indiana State University, Terre Haute, Ind.). The embryos used in preparation of CE cell cultures and the chicks used for inoculation were leukosis-free (SPAFAS) white leghorn chickens. Secondary CE cell cultures were maintained with Eagle's minimal essential medium (MEM) supplemented with 5% newborn calf serum (Flow), 10% tryptose phosphate and 25 µg/ml gentamycin.

Isolation of transformed cells. One-day-old chickens were inoculated with 0.2 ml of a 20% liver homogenate obtained from a chicken moribund with reticuloendotheliosis. Cells were obtained from the bone marrow of the femur of these chickens when they showed symptoms of reticuloendotheliosis. The femur was severed and the cells were aspirated with the medium described below. The cells were then transferred to tissue culture flasks (Falcon) and grown in MEM supplemented with 10% fetal calf serum, 1% CE extract prepared from SPAFAS embryos and 25 µg/ml gentamycin (Schering).

Preparation and purification of labeled virus. All isotopes were obtained from Amersham/Searle. MEM with dialyzed calf serum was used for labeling virus preparations with 3H-uridine (20 µCi/ml; 25 Ci/mmole), Phosphate-free MEM was used for labeling with 32P (10 µCi/ml; 98 Ci/mg P), and leucine-free MEM for 3H-leucine (10 µCi/ml; 46 Ci/mmole) and 14C-leucine (3 µCi/ml; 348 mCi/mmol) labeling. Virus particles labeled for 48-72 h were recovered from the culture fluids by differential centrifugation and centrifuged in a preformed 15-45% w/w linear sucrose gradient for 4 h at 35,000 rpm in an SW41 rotor (Beckman).

RNA extraction and sedimentation. Virus from the bone marrow cells and REV propagated in CE cell cultures were mixed prior to extraction of the RNA. RNA was extracted 3× with double-distilled phenol and sodium dodecyl sulfate (SDS) using polyvinyl sulfate and 0.5% diethylpyrocarbonate (Baycovin) to inhibit RNase. (Baycovin was a gift from Mobay Chemical Co., Pittsburg, Pa.). The RNA was precipitated overnight at −20°C with 2-4 vol of 95% ethanol. The RNA was then layered on a preformed 5-20% w/w linear sucrose gradient and centrifuged at 40,000 rpm in an SW50.1 rotor (Beckman) for 1 h at 15°C. Fractions were precipitated with 5% cold trichloroacetic acid (TCA) and counted in a Nuclear Chicago Mark I liquid scintillation counter using 10 ml of Bray's scintillation fluid [14].
Polyacrylamide gel electrophoresis. The virus preparations were prepared for SDS-PAGE by digestion with 0.5% SDS, 0.5% 2-mercaptoethanol and 4 M urea [15] and dialyzed to remove soluble label. 10% polyacrylamide gels (100 x 5 mm) with bisacrylamide were prepared with Hay's buffer [16]. Samples were electrophoresed for 8 h at 8 mA per gel, and 1-mm slices were obtained with a transverse slicer (Diversified Scientific Instruments, Inc.). The slices were swollen overnight in 0.5 ml water before determining radioactivity.

Results

Although transformed cells are present in livers and spleens recovered from REV-infected moribund chicks [13], previous efforts at growing these cells in culture have not been successful. However, we have now obtained from the femur of REV-infected chicks bone marrow cells (RE-bone marrow) with characteristics indicating that they are transformed.

The original cell culture obtained from the bone marrow of an infected bird has been maintained through more than 30 serial passages. Passages are done every 3-5 days by inoculating 6 ml of a dense culture (2.0 x 10^6 cells/ml) in 75 ml of fresh MEM. The cells grow in suspension as loose aggregates (fig. 1A). The doubling time of these cells is 10-14 h, and they attain a cell density of 3 x 10^6 cells/ml. Cells from the bone marrow of uninfected chicks of the same age did not divide and thus could not be subcultured. Wright's stain (fig. 1B) indicates that the cells are polymorphous and that the nucleus comprises most of the cell volume. Multinucleated cells are frequently observed.

To determine if these cells were tumorigenic, 10^5 cells were inoculated into the wing web of day-old chicks. Tumors appeared at the site of inoculation 3 days later. The chicks died within 8 days after inoculation. Autopsy revealed enlarged, necrotic livers and spleens which are characteristic of REV infection. Cells isolated from the wing web tumors had morphology and growth characteristics similar to those of the bone marrow cells used as the inoculum. Bone marrow cells from uninfected chicks did not induce wing web tumors.

We determined that these cells were producing extracellular virus particles by inoculating cell-free filtrates from the culture fluids into day-old chicks. Cell-free filtrates were prepared by filtration through a nucleopore filter (pore size, 1.0 μm). Eight days after inoculation, all the chicks (10/10) were dead. Autopsy revealed characteristic REV lesions.

We attempted to obtain labeled virus particles for biochemical characterization by incubating the RE-bone marrow cells in the presence of ^3H-uridine, ^3H-leucine or ^32P for 48-72 h. The culture fluids were harvested, and
Fig. 1. A Suspension culture of bone marrow cells obtained from REV-infected, moribund chicks. × 58. B Wright's stain of RE-bone marrow cells. × 720.
Fig. 2. Sucrose gradient purification of particles from RE-bone marrow cells. The virus was centrifuged in a 15-45% w/w linear sucrose gradient for 4 h at 35,000 rpm in an SW41 rotor (Beckman). • = \textsuperscript{3}H-uridine-labeled virus obtained from cocultivation of RE-bone marrow cells and CE cells. \(5 \times 10^6\) secondary CE cells were inoculated with \(10^6\) RE-bone marrow cells in a tissue culture flask and labeled for 48-72 h before harvesting. ○ = \textsuperscript{3}H-uridine-labeled virus obtained from RE-bone marrow cells. \(10^6\) RE-bone marrow cells were labeled and grown for 48-72 h before harvesting.

virus particles were concentrated by differential and sucrose gradient centrifugation. In all cases the amount of label incorporated into particles banding at the buoyant density of REV was insufficient to permit further characterization. The amount of infectious virus released by the RE-bone marrow cells was low, as indicated by low LD\textsubscript{50} titers (10\textsuperscript{3}/ml). Since adequate amounts of labeled REV can be obtained by infecting CE cell cultures, we cocultivated an equivalent number of RE-bone marrow cells with preformed CE cell monolayers. Figure 2 indicates that a substantial increase in label incorporation into virus particles was achieved by cocultivation. This figure compares the amount of label incorporated into virus particles by incubation of RE-bone marrow...
REV Transformed Cells

Fig. 3. RNA species present in virus particles obtained from cocultivation of RE-bone marrow cells and CE cells. RNA was extracted using SDS-phenol and precipitated with 4 vol of 95% ethanol. The RNA was centrifuged in a 5-20% w/w linear sucrose gradient for 1 h at 40,000 rpm in an SW50.1 rotor (Beckman) at 15°C. ○ = 3H-uridine-labeled virus RNA from RE-bone marrow cells. ● = 32P-labeled virus RNA marker from CE cells infected with an REV liver homogenate.

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from cocultivation cultures co-sedimented with the 64S and 4S marker RNA obtained from REV.

Since REV does not contain the ATV group-specific (gs) antigen [10, 11], we performed complement fixation tests [17] with the virus particles concentrated from cocultivation cultures by sucrose gradient centrifugation. ATV gs antigen was not detected. Similarly concentrated RSV preparations were positive at a 1:32 dilution.

Consistent with the absence of the ATV gs antigen, it has been shown that the major nonglycosylated polypeptide of REV does not co-migrate with the major gs antigen containing polypeptide of RSV [4, 5]. The major nonglycosylated polypeptide of COFAL-positive viruses co-migrates [18, 19]. We analyzed the polypeptide composition of the virus obtained by cocultivation and compared it by PAGE to that of marker RSV and REV obtained from REV liver homogenate-infected CE cell cultures. Figure 4A demonstrates that the major nonglycosylated polypeptide of these virus particles co-migrates with that of the marker REV but does not migrate with the corresponding polypeptide of RSV (fig.4B). These results indicate that the RE-bone marrow cells are producing REV and that avian leukosis viruses have not been induced in detectable levels.

Discussion

It has been suggested that REV induces a hyperplastic rather than a neoplastic disease [20, 21]. However, Sevoian and co-workers [1] have described the disease as similar to those caused by the leukosis virus complex. The induction of tumors in the wing web following inoculation with REV liver homogenates suggests that reticuloendotheliosis is a neoplastic disease [13]. The isolation of bone marrow cells from REV-infected birds which induce wing web tumors (indicating that they are transformed) confirms that REV is an oncogenic virus.

Fig. 4. PAGE of disrupted virus particles obtained from RE-bone marrow cells cocultivated with CE cells. REV from CE cells infected with an REV liver homogenate and Bryan RSV (RAV-1) were used as markers. Virus particles were digested in a solution containing 0.5% SDS, 0.5% 2-mercaptoethanol and 4.0 M urea. This preparation was electrophoresed in 10% SDS-polyacrylamide gel for 8 h at 8 mA. Gels were frozen at -70° and sliced on a transverse gel slicer (Diversified Scientific). A = 3H-leucine-labeled virus from cocultivation of RE-bone marrow cells and CE cells. • = 14C-leucine-labeled REV from CE cells infected with an REV liver homogenate. B = 3H-leucine-labeled virus from cocultivation of RE-bone marrow cells and CE cells. • = 14C-leucine-labeled Bryan RSV (RAV-1).
REV Transformed Cells

**A**

![Graph A](image)

**B**

![Graph B](image)
The REV transformed bone marrow cells morphologically resemble those transformed by avian myeloblastosis virus (AMV). However, these cells differ in their division time and the type of viruses which they produce. The division time of transformed avian myeloblasts is 48 h [22] and may be as long as 7 days in some cases [23]. By contrast, the REV-transformed bone marrow cells have a division time of 10-14 h. The virus produced by transformed myeloblasts is AMV, a member of the avian tumor virus complex. The virus produced by the REV-transformed bone marrow cells induces reticuloendotheliosis and has other properties indicating that it is REV and not a member of the avian tumor virus complex.

Our inability to obtain substantial amounts of labeled virus particles from the culture fluids of RE-bone marrow cell cultures with a variety of isotopes suggests that low amounts of virus particles are produced by those cells. Alternatively, virus particles may be produced in substantial levels but most may remain adsorbed to the cell surface.

Although low LD₅₀ titers were obtained from the culture fluids of the RE-bone marrow cells, these virus particles appear to be more oncogenic for newborn chicks than REV which has been propagated in CE cell cultures. The reason for this remains to be determined. The loss of oncogenic potential of REV propagated in CE cell cultures has been reported [13, 24]. This loss may be due to selective loss of an oncogenic variant in the virus preparation or to modification of the virus in CE cell cultures. Studies are now in progress to elucidate the molecular basis of the loss of oncogenicity. In addition, we are attempting to determine the infectious unit to particle ratio of REV from RE-bone marrow cells and REV propagated in CE cell cultures.

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References


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