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PRESENCE OF RETROVIRUS IN THE B95-8 EPSTEIN-BARR VIRUS-PRODUCING CELL LINE FROM DIFFERENT SOURCES

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SUMMARY

The B95-8 cell line, a widely used source of highly transforming Epstein-Barr virus (EBV), obtained from the laboratory of origin, harbored an infectious retrovirus. This retrovirus generally resembled the Type D retroviruses structurally and developmentally and like the Type D retroviruses preferred Mg^{2+} to Mn^{2+} in its RNA-directed DNA polymerase reaction. Evidence for the presence of retrovirus was found in B95-8 cultures from two other sources within the United States, either by assay for polymerase or by electron microscopy. Comparison of two B95-8 cell lines showed cytogenetic differences as well as differences in retroviral activities. The results suggest that any B95-8 culture should be tested for the presence of retrovirus before its use as a source of EBV.

Key words: retrovirus; marmoset cells; EBV-producer.

INTRODUCTION

B95-8 is the designation of a continuous, cotton-top marmoset cell line transformed by Epstein-Barr virus (EBV) (12). A human lymphoblastoid cell line from a patient with transfusion-induced infectious mononucleosis was the source of EBV used to transform the marmoset leukocytes of B95-8 (2). Epstein-Barr virus produced by B95-8 has a relatively high transforming capacity as measured by the immortalization of human lymphocytes from umbilical cord blood (13). Because of the transforming nature and the comparatively high yields of EBV from B95-8, this culture has been a widely used source of EBV in recent years.

Electron microscopic examination of thin sections of B95-8 cell pellets unexpectedly revealed structures characteristic of retroviruses. We present a partial characterization of a Type D retrovirus detected in B95-8 cultures obtained from the laboratory of origin as well as evidence for the presence of retrovirus in B95-8 from two other sources within the United States.

MATERIALS AND METHODS

Cell lines. The following cell lines from the indicated sources were used in this study: EBV-producing marmoset lymphocyte cultures, B95-8 (M) (G. Miller, Yale University, New Haven, CT); B95-8 (LS) (Life Sciences, St. Petersburg, FL); B95-8 (NERPC) (L. Falk, New England Regional Primate Center, Southboro, MA); bat lung culture, M241/88 MC1-33, producing Type D langur retrovirus (P0-1-Lu) (21) (Meloy Labs, Springfield, VA); human rhabdomyosarcoma, A204 (4) (Hazleton Labs, Vienna, VA); a mouse cell line, 3T3/NIH C1-1, producing Type C Moloney leukemia virus (MoMULV) (Hung Fan, Salk Institute, La Jolla, CA); EBV-containing human lymphoma cultures, RAJ1 (17) (American Type Culture Collection, Rockville, MD); and P3JHR-1 (P3HR-1) (8) (Gull Labs, Inc., Salt Lake City, UT). The defined media (GIBCO, Grand Island, NY) for these cell lines

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were: RPMI 1640 for lymphocyte cultures, Eagle's minimum essential medium with or without nonessential amino acids for M241/88 MC1-33, modified Dulbecco's medium (1 g D-glucose/l) for A204, and McCoy 5A medium for 3T3/NIH C1-1. Cell line 3T3/NIH C1-1 received additional L-glutamine (1 ml, 200 m*M*/100 ml medium). Heated (56° C, 30 min) bovine newborn calf serum (Reheis Chemical Co., Phoenix, AZ) constituted 10% (15% fetal bovine serum (FBS) for 3T3/NIH C1-1) of the growth medium of these cell lines.

To detect mycoplasma, cell suspensions were inoculated onto agar medium (11) and incubated anaerobically at 37° C or thin sections of cell pellets were examined by electron microscopy.

Electron microscopy. Cell pellets were fixed in 3% glutaraldehyde in phosphate buffer, postfixed in 1% osmium tetroxide in phosphate buffer, stained with 0.1% aqeous uranyl acetate solution, dehydrated in increasing concentrations of ethanol, and embedded in Araldite 502 (CIBA Co., Inc., Summit, NJ). Thin sections were stained with 2% uranyl acetate and lead citrate (19).

Preparatory to negative staining, spent culture medium was clarified at 600 ×*g* and then centrifuged for 45 min at 82,700 ×*g*. The virus pellet was resuspended in a minimal volume consisting of equal parts deionized water, 1% bovine albumin, and 4% potassium phosphotungstate and placed on 400-mesh copper grids coated with parlodion and carbon. Specimens were examined with the Siemens Elmiskop I.

Immunofluorescence. Cell suspensions were applied to glass slides as small drops, air dried at 37° C and fixed 7 min in acetone at −20° C. In the anticomplement fluorescence test to detect EBV nuclear antigen (EBNA) (18), guinea pig complement and antiguinea pig C3 conjugated with fluorescein isothiocyanate (Cappel Labs, Cochranville, PA) were used. Viral capsid antigen (VCA) (7) was detected by the indirect method with fluorescein isothiocyanate-conjugated anti-human IgG (Fc fragment) (Cappell Labs). Human sera with or without EBV antibody were selected using Raji and P3JHR-1 cell lines.

Metabolic labeling of virus. At 70% of confluence, cell cultures were labeled with 20 μCi/ml [^{3}H]-8-leucine (40 to 60 Ci/μmol) (New England Nuclear, Boston, MA) in growth medium lacking leucine and supplemented with 10% dialyzed fetal bovine serum (GIBCO). Cultures were labeled for 12 h.

Sucrose gradient banding of virus. Five hundred milliliters of spent culture medium was clarified 10 min at 8000 ×*g*, and virus was pelleted for 4 h at 52,000 ×*g*. The virus pellet was resuspended in a minimum amount of TNE buffer (0.01 *M* Tris-HCl, 100 m*M* NaCl, 0.001 *M* ethylene-diaminetetraacetic acid, pH 8.0) and applied to a 10 to 65% sucrose gradient. Virus was banded by centrifugation for 2 h at 40,000 ×*g*. One-milliliter gradient fractions were precipitated onto filters (Gelman Sciences, Inc., Ann Arbor, MI) with 25% trichloroacetic acid, and the radioactivity of each fraction was determined by liquid scintillation counting as described by Syrewicz et al. (20).

RNA-Directed-DNA polymerase. Twenty-five microliters of virus concentrated as above was lysed in a solution containing 0.2% Triton X-100, 0.0024 Tris-HCl, pH 8.2, and the polymerase was assayed as described by Todaro et al. (21).

Rate zonal sedimentation of viral RNA. B95-8 (M) and B95-8 (LS) cultures containing approximately equivalent numbers of cells were labeled for 48 h with [^{3}H]uridine in fresh medium containing 2% FBS. Concentration of virus, extraction of viral RNA, rate zonal centrifugation of extracted native RNA, and calculation of sedimentation coefficient were carried out as described by Nash et al. (14). Radioactive P0-1-Lu virus from M241/88 MC1-33 was prepared and analyzed similarly.

Infectivity. Cell line A204 was inoculated with the B95-8 (M) retrovirus as described below. Forty-eight to seventy-two hours after the last medium change, B95-8 (M) cells were separated from spent culture medium by centrifugation at 600 ×*g* and filtration of the medium through a 0.45 μm filter, followed usually by filtration through a 0.22 μm filter. A204 cultures were exposed for several days to large volumes of undiluted, filtered medium containing the B95-8 (M) retrovirus and then were maintained by regular medium replacement. After at least 1 mo, infection was monitored either by polymerase assay and electron microscopic observations or by the latter method alone. Cell line A204 was inoculated with cell-free P0-1-Lu from M241/88 MC1-33 and monitored similarly.

Cytogenetic analysis. Exponentially growing cultures of B95-8 (M) and of B95-8 (LS) were treated with Colcemid (0.05 μg/ml) for 1 h. Cells were dislodged by scraping with a rubber policeman, allowed to swell in a hypotonic solution of sodium citrate (1.0% at room temperature) for

20 min, and then fixed in methanol-acetic acid mixture (3:1) for 15 min. After washing twice in the fixative, cells were air dried on acetone-cleaned slides.

For each cell line, 20 conventionally Giemsa-stained metaphase spreads were used to determine the stem line chromosome number. For the staining of nucleolus organizer regions (NOR), a modified Ag-NOR procedure was used (15). No other banding technique was applied to such preparations.

Results

Structure and development of the B95-8 (M) retrovirus. Initially, the B95-8 (M) retrovirus was found in thin sections of B95-8 (M) cells by electron microscopy several months after cultivation of B95-8 (M) began in this laboratory. Intracytoplasmic Type A particles (Fig. 1), budding Type A particles (Fig. 2), and extracellular mature virions, containing a usually centric, electron-dense core surrounded by an intermediate membrane (Fig. 3) were observed. Extended plasma membrane buds sometimes encased multiple Type A particles (Fig. 2). Rectangular or bar-shaped nucleoids (Fig. 3) were very rare in virions from B95-8 (M) and from A204 cultures. In this aspect, the B95-8 (M) retrovirus differed from the Mason-Pfizer monkey virus (MPMV), which when derived from various cell lines, including A204, often contains tubular, bar-shaped, or triangular nucleoids (5,10). Mature virions measured approximately 135 nm in diameter. Irregularly formed virions were common. No definite evidence for the presence of projections or spikes was found in negatively stained preparations of unpurified virus (Fig. 4). Similar retroviral structures also were seen in a second B95-8 culture received from G. Miller and prepared for electron microscopy immediately upon receipt without intervening cultivation in this laboratory. Subsequent analyses were done on the isolate from the first culture of B95-8 provided by G. Miller. Based on structural development, the B95-8 (M) retrovirus generally resembled members of the D group of retroviruses, e.g., MPMV (5,10), squirrel monkey retrovirus (SMRV) (6), and P0-1-Lu (21).

Structurally similar retrovirions were found in two separate B95-8 cultures from G. Miller and in B95-8 (NERPC), but retrovirions were never found in B95-8 (LS) despite the presence of polymerase described below. Epstein-Barr virus was observed infrequently in the B95-8 cultures (Fig. 5).

RNA-directed DNA-polymerase activity. Polymerase assays of virus pelleted as above from B95-8 (M) and from B95-8 (LS) confirmed the presence of a retrovirus in B95-8 (M) and suggested its presence in B95-8 (LS). Although many separate examinations of B95-8 (LS) by electron microscopy never revealed physical particles, assays repeatedly indicated polymerase activity approximating that measured in B95-8 (M). The assay of polymerase activity in B95-8 cultures, inoculated cultures, and control cultures is shown in Table 1.

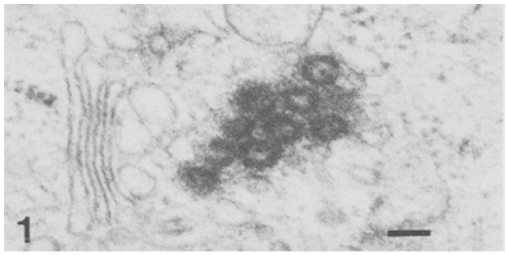
Previous studies reported a Mg^{2+} preference for the polymerase of SMRV (6), P0-1-Lu (21), and MPMV (1). The polymerase of P0-1-Lu exhibited the expected Mg^{2+} preference, using 10 m*M* Mg^{2+} and 10 m*M* Mn^{2+} in these assays. The polymerase of C-type MoMuLV also showed its expected Mn^{2+} preference. The polymerase of the B95-8 (M) retrovirus preferred Mg^{2+} to Mn^{2+} but at a lower concentration than P0-1-Lu. These data showed further similarities between the B95-8 (M) retrovirus and the Type D retroviruses.

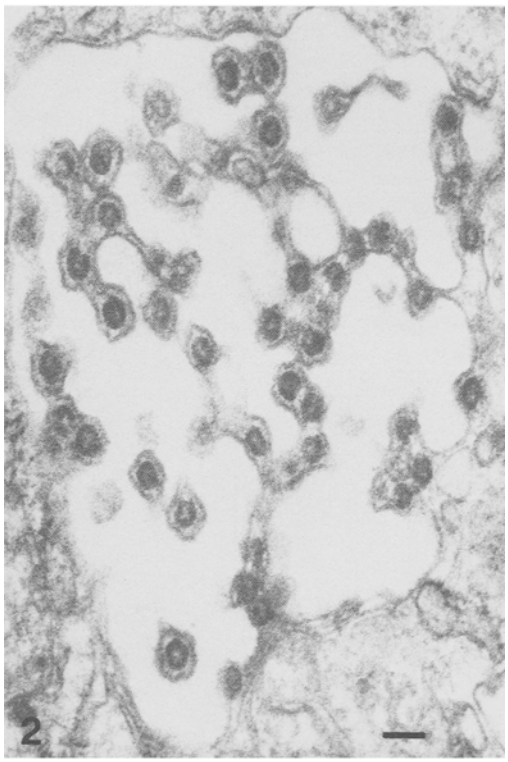
Buoyant density. The sedimentation profiles, broadly distributed around a peak of radioactivity at 1.15 g/cm^3, which is characteristic of retroviruses, further indicated the retroviral nature of the B95-8 (M) isolate. Figure 6 illustrates the sedimentation profile of concentrated virus derived from A204 infected with the B95-8 (M) retrovirus. A more diffuse peak was observed in the profile of pelleted particulates from the B95-8 (LS) culture and no peak of radioactive virus was observed in the profile from A204 inoculated with cell-free medium from B95-8 (LS). When alternate fractions of the gradient were assayed for polymerase activity, no appreciable activity was found. Diffuse banding during the influence of osmotic and centrifugal stresses might have been due to virion fragility, particularly in view of the reported fragility (10) of the prototype D retrovirus, MPMV, during centrifugation in a sucrose density gradient. In contrast, the profile of P0-1-Lu showed a sharp peak of the polymerase activity measured in the same gradient.

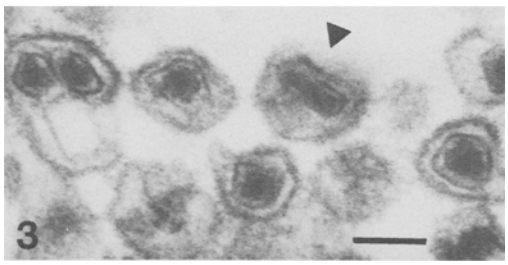
Sedimentation coefficient of B95-8 (M) retrovirus RNA. Figure 7 compares the rate zonal sedimentation profile of RNA from the B95-8 (M) isolate with that of a known Type D retrovirus, P0-1-Lu. The native genomic RNA of the B95-8 (M) isolate exhibited a sedimentation coefficient of 58S, which was similar to that of PO-1-Lu and

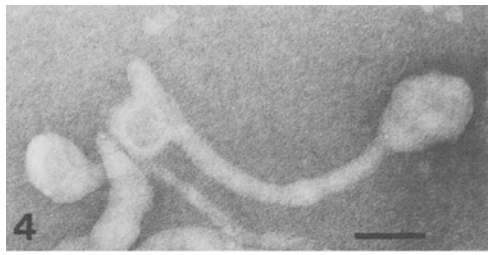
suggestive further of retroviral origin. No significant amount of 58S RNA was released from the B95-8 (LS) culture (Fig. 7 *A*) despite a level of polymerase activity in B95-8 (LS) comparable to that in B95-8 (M) (Table 1).

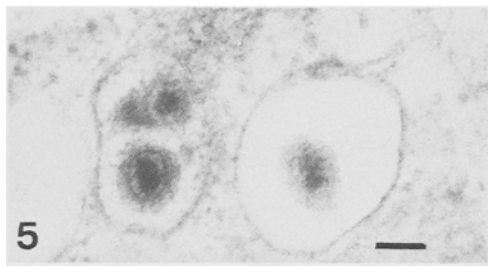
Infectivity. Cell line A204, which supports the replication P0-1-Lu, MPMV, and SMRV, also was susceptible to the B95-8 (M) retrovirus. Budding virions were detected in A204 cultures after inoculation with cell-free, spent medium from B95-8 (M) in three of five attempts. Two of the cultures with budding virions were tested for polymerase activity and found to contain activity significantly above background. Contrastingly, in three of three inoculations of A204 cultures with cell-free medium from B95-8 (LS), no budding virions were found. Two of these cultures were tested for polymerase activity; no significant ac-











FIGS. 1–5. Bars represent 100 nm.

FIG. 1. Retroviral forms from B95-8 (M). Intracytoplasmic Type A particles. ×56000.

FIG. 2. Retroviral forms from B95-8 (M). Budding and extracellular enveloped Type A particles. ×54000.

FIG. 3. Retroviral forms from B95-8 (M). Extracellular mature virions. Virion with bar-shaped nucleoid (*arrow*). ×94000.

FIG. 4. Retroviral forms from B95-8 (M). Negatively stained, tailed retroviral form from B95-8 (M). ×92000.

FIG. 5. Enveloped EB virion in intracytoplasmic vacuole from B95-8 (LS). ×68000.

TABLE 1

RNA-DIRECTED DNA-POLYMERASE ACTIVITY OF THE B95-8 (M) RETROVIRUS AND OTHER RETROVIRUSES IN DIFFERENT CELL LINES

Viruses and Cell Lines	[^{3}H]TMP Incorporation (cpm × 10^{-2})			
	2.5 m*M*		10 m*M*	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
MoMuLV in 3T3/NIH C1-1	30	45	30	55
P0-1-Lu in M241/88 MCL-33	35	40	50	29
P0-1-Lu in A204			40	30
B95-8 (M)	50	27	27	31
B95-8 (LS)			28	30
A204			10	9
B95-8 (M) retrovirus in A204			140	140
B95-8 (LS) retrovirus in A204			10	10
Deionized H_2O	8	9	8	7

tivity was detected. Immunofluorescence tests for EBNA and VCA were negative in A204 producing the B95-8 (M) retrovirus, thus assuring that the procedures to produce a virus inoculum free of B95-8 (M) cells were effective.

Identity of B95-8 cell lines. Because of the presence and the dissimilar expression of retrovirus (es) in the B95-8 cultures from different sources, cytogenetic analysis was done to ascertain the identity of these cell lines. In Line B95-8 (M), the predominant chromosome number was 45, whereas in B95-8 (LS) the number was 46. In the majority of silver stained metaphase spreads of B95-8 (M), the number of NOR-bearing chromosomes was four (Fig. 8). In B95-8 (LS) metaphase spreads, a maximum of six NOR-bearing chromosomes were noticed (Fig. 9).

The karyotype (2N=46) of the cotton-top marmoset, *Saguinus oedipus*, has two pairs of acrocentric autosomes with secondary constriction in the proximal end of the long arm (9). Based on the characteristic features of these marker chromosomes (NOR-bearing autosomes) it was concluded that the two cell lines, B95-8 (M) and B95-8 (LS), seemed to be variants of marmoset origin.

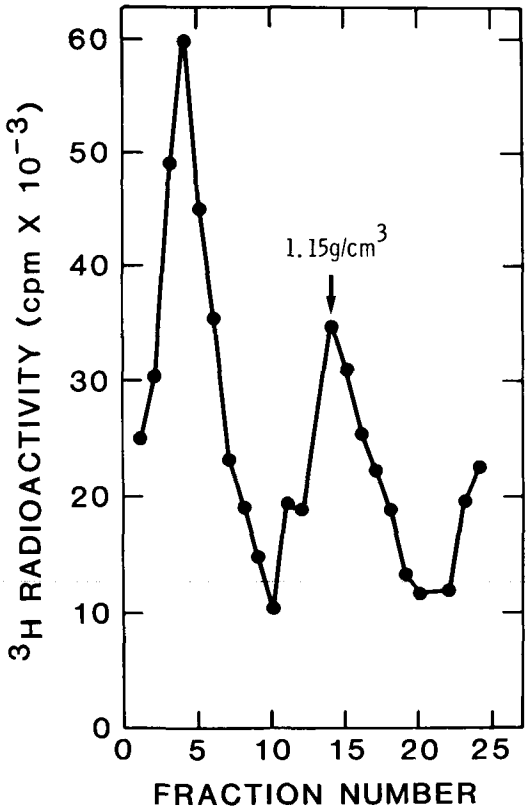


FIG. 6. Buoyant density in sucrose gradient of B95-8 (M) retrovirus derived from A204.

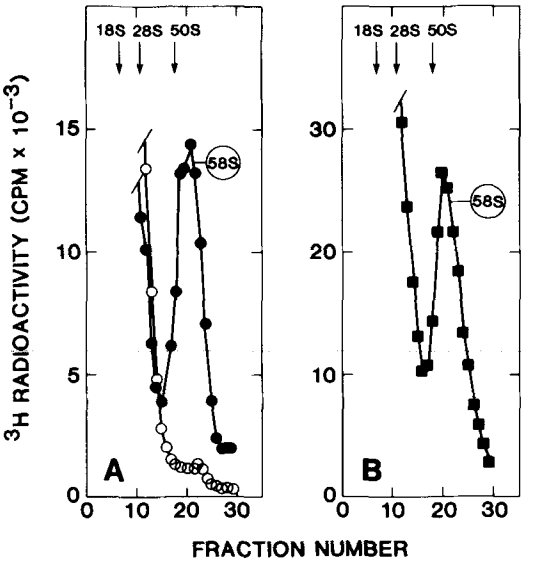


FIG. 7. Rate zonal sedimentation profiles of RNA released by *A*, B95-8 (M) cells, *solid circles;* B95-8 (LS) cells, *open circles*. *B*, P0-1-Lu.

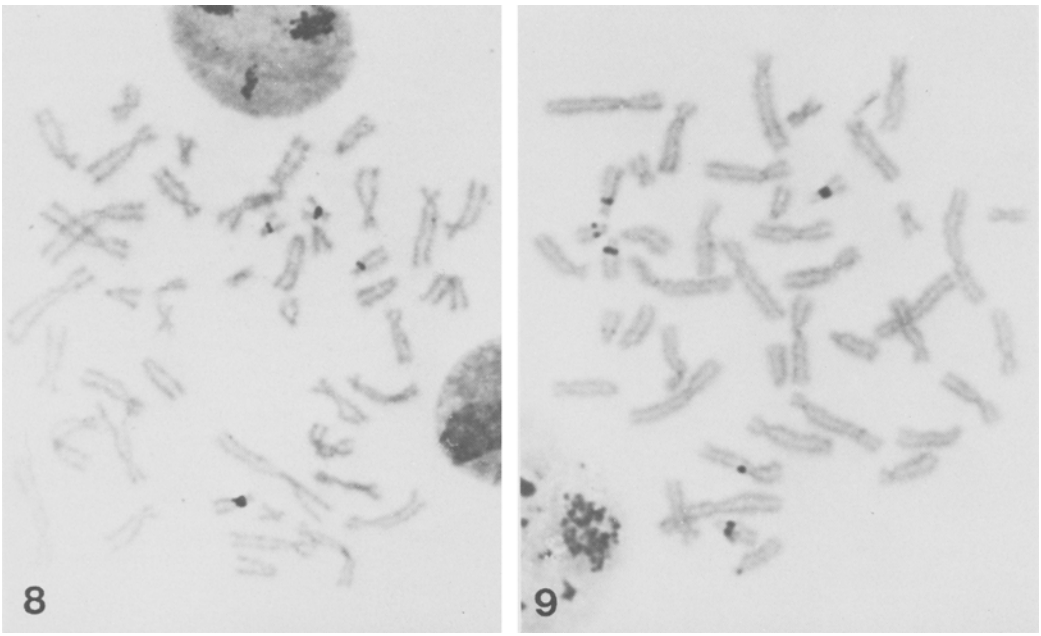


FIG. 8. Metaphase plate of B95-8 (M).

FIG. 9. Metaphase plate of B95-8 (LS).

Mycoplasma structures were observed in B95-8 (NERPC), and the chromosomal constitution of this B95-8 line was not analyzed. However, consistent with its presumed identity, B95-8 (NERPC) contained EBV-like virions. Mycoplasma were not detected in the other two B95-8 lines by electron microscopy or by the biological method. Immunofluorescence tests for EBV-specific antigens, EBNA and VCA, in B95-8 (M) and in B95-8 (LS) were positive.

DISCUSSION

Evidence was found for the presence of retrovirus in B95-8 cultures from three separate sources within the United States. The degree of expression of retrovirus was different in two cell lines, B95-8 (M) and B95-8 (LS), compared in detail. The only indication of retroviral presence in B95-8 (LS) was polymerase activity in highly concentrated pellets from spent media; however, it was concluded by several criteria that a retrovirus was present in B95-8 (M). The presence of polymerase and the absence of infectivity suggested defectiveness of the putative retrovirus in B95-8 (LS).

During the latter part of this investigation, Popovic et al. (16) reported a Type D retrovirus, closely related to SMRV, in a human myeloma line. It was observed secondarily that the same virus was present in B95-8 from Swedish but not other sources, including sources within the United States. Presumably, the B95-8 (M) Type D retrovirus is the same virus. Our result confirmed and extended observations of retroviral contamination in the B95-8 cell line. Thus, retroviral contamination is not confined only to Swedish sources of B95-8 but was found to be more widespread.

Cytogenetic analysis showed further that sublines of B95-8 now exist. B95-8 (M) and B95-8 (LS) differed in number of chromosomes and of nucleolus organizer regions. In view of genotypic changes in B95-8 cultures, differences in expression of a commonly present retrovirus(es) and differences in EBV antigen expression (Tumilowicz, unpublished results) may not be unexpected.

Conceivably, the retrovirus in B95-8 may be a variant of SMRV, an indigenous marmoset Type D virus, or a Type D retrovirus of human origin present in the inoculum from human cells used to infect B95-8 cells initially. Related to the latter possibility is the finding by Chiu et al. (3) that cloned SMRV DNA hybridizes with human DNA, suggesting among other possibilities the existence of a human analog of SMRV. Based on structural considerations, the B95-8 (M) retrovirus did not seem identical to MPMV.

Regardless of the origin of the retrovirus(es) in B95-8, the combined data suggested that any B95-8 culture, irrespective of source, should be monitored for presence of retrovirus before use as a source of transforming EBV. In addition to operational complications introduced by a contaminating virus, synergistic effects, which may affect the transformed state induced by EBV, must be considered.

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