Biological Characterization of an Epstein-Barr Nuclear Antigen-positive American Burkitt's Tumor-derived Cell Line

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SUMMARY

Two distinct cultures derived from a lymphoid cell line designated NAB were characterized immunologically, morphologically, and cytogenetically. Both cultures were positive for Epstein-Barr nuclear antigen. NAB I cultures were negative for virus capsid antigen and early antigen and were not affected by treatment with 5-iododeoxyuridine. NAB II cultures were positive for virus capsid antigen and early antigen, which increased with 5-iododeoxyuridine treatment. Both cultures were superinfected with virus prepared from P3HR-1 cells. Cell-free virus concentrates prepared from both cultures were inactive for transformation and infectivity.

NAB I and NAB II cells were lymphoid as determined by light and electron microscopy. NAB II cells showed morphological alterations characteristic of herpes infection. 5-Iododeoxyuridine-treated cells from both cultures revealed ultrastructural characteristics of cells infected with herpesviruses but without virus particles. In addition, the induction of tubuloreticular structures within the endoplasmic reticulum was observed.

Cytogenetic analysis of both cultures revealed a rearranged chromosome 14 and several other chromosome aberrations, three of which may be used as a reliable means of identifying NAB cultures.

INTRODUCTION

EBV\(^3\) a DNA herpesvirus, was first morphologically identified by electron microscopy in lymphoblasts derived from an African Burkitt's lymphoma (12). Since that time, several lymphoid cell cultures containing EBV have been established from this same type of African Burkitt's lymphoma tumor (20). Although relatively rare, cases of typical Burkitt's lymphoma have been reported in American children (1, 2, 4, 7, 44). Few studies, however, have been published on the ultrastructure of American Burkitt's tumor cells (8, 47). In contradistinction to several African Burkitt's lymphoma cell lines, there was only 1 report of herpes-like particles being observed by electron microscopy in suspension cultures of cells derived from an American lymphoma (43); therefore, a more complete biological and morphological characterization of a cell line derived from an American Burkitt's lymphoma was undertaken (17).

MATERIALS AND METHODS

Cell Culture and I\(\text{UdR}\) Treatment. The NAB (17), Raji (13), and P3HR-1 (26) cultures were obtained from Dr. G. Pearson, NIH, and the B-95-8 culture (37) was obtained from Dr. G. Miller, Yale University. NAB cultures were received at Frederick Cancer Research Center approximately 2 months after the establishment of the cell line (17). These cultures were studied over a 2-year period. The cells were grown in suspension culture consisting of Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 \(\mu\)g/ml), and atolin (60 \(\mu\)g/ml). Cultures were monitored daily for cell viability by the trypan blue exclusion technique. When the cultures contained 1 to 2 \(\times\) 10\(^6\) viable cells/ml, they were split 1:2 with fresh medium, and incubation at 37\(^\circ\) was continued.

In an attempt to enhance virus expression, we treated NAB cultures with medium containing 10 \(^{-5}\) \(\text{M I\(\text{UdR}\) (Sigma Chemical Company, St. Louis, Mo.) for 4 days. Control cultures were maintained as described above. After 4 days duplicate cell pellets were processed for immunofluorescence, virus infectivity, and electron microscopy.

Virus Preparation. Culture fluids were clarified by centrifugation at 100 \(\times\) 10 min. The cell-free supernatant was again centrifuged (1500 \(\times\) g for 30 min), and a 100-fold virus concentrate was prepared from the clarified and filtered (0.8-\(\mu\)m Millipore filter) fluid by centrifugation at 35,000 \(\times\) g for 90 min.

Immunofluorescence Assay. The percentage of antigen-positive cells was determined by IFL (24) with EBV-positive and EBV-negative human sera and goat anti-human immunoglobulin labeled with fluorescein isothiocyanate (Hyland Laboratories, Costa Mesa, Calif.). The EBV sera contained high VCA and low EA antibodies to EBV as determined by testing with P3HR-1 and superinfected Raji cells, respectively. EBNA in human cord leukocytes and NAB cells was
determined by the IFL method described by Reedman and Klein (49), with Raji cells as the positive control.

**Infectivity Testing.** Infectivity was determined by super-infection of Raji cells (25). Raji cells diluted to 5 × 10⁶ cells/ml were grown in 5 ml of Roswell Park Memorial Institute Tissue Culture Medium 1640 plus 10% fetal bovine serum in T30 flasks. The flasks were serially inoculated with 10-fold dilutions of virus and were incubated at 35° for 4 days. The superinfected cells were removed from the culture medium by centrifugation, washed with Dulbecco’s phosphate-buffered saline (9), and spotted on Teflon-coated slides. The slides were air-dried, acetone fixed, treated with positive and negative EBV sera, and then examined for EA by IFL. The infectivity titer was recorded as the reciprocal of the highest virus dilution giving 0.5% IFL-positive cells. The presence of transforming EBV (39) was confirmed in 5% CO₂ for 7 days for EBNA testing.

**Cyto genetic Methods.** Samples of the NAB I and II cultures were harvested for chromosome studies approximately 12 to 24 hr after splitting. Cells were arrested in metaphase by the addition of 0.2 µg of Colcemid per ml of culture fluid for 20 min immediately before being exposed to hypotonic treatment with 0.075 M KCl. After 20 min in hypotonic KCl, the cells were fixed twice in methanol:acetic acid (3:1), spread on slides wet with the same fixative, and air-dried.

Slides chosen for chromosome banding were dried in a vacuum desiccator for 4 to 24 hr, treated with 0.03 to 0.05% trypsin (Difco Laboratories, Detroit, Mich.) (50), and stained for 32 min in buffered 2% Giemsa solution. Thirty metaphase preparations from each culture were counted and analyzed. Of these, 12 spreads from each culture were photographed through a ×100 objective with Kodak high-contrast copy film, and they were karyotyped according to the recommendation of the Paris Conference, 1971 (3).

**Preparation of Cells for Light and Electron Microscopy.** Cells to be examined by electron microscopy were pelleted in culture fluid at 200 × g for 15 min. The supernatant fluid was removed, and the cell pellet was fixed with one-half-strength Karnovsky’s fixative (29) for 1 to 2 hr at 4°. After primary fixation the cell pellet was rinsed several times in 0.1 M sodium cacodylate buffer, pH 7.2, and stored in cacodylate buffer containing sucrose for further processing. The cell pellets were postfixed in 1% osmic acid (5) and stained en bloc with uranyl acetate (30). Specimens were dehydrated through a graded ethanol series, cleared in propylene oxide, and embedded in Maraglas (15). Thick plastic sections were stained with toluidine blue and pyronin Y (52) and were examined by light microscopy. Representative fields were photographed. Silver/gold thin sections were cut with an LKB Ultratome with the use of diamond knives. Sections were doubly stained with uranyl acetate and then lead citrate (14) and were examined in a Siemens Elmiskop I or a Hitachi HU-12 electron microscope.

**RESULTS**

NAB early-passage cells were EBNA positive and VCA and EA negative (17). After several months in suspension culture, a culture arose that was EA and VCA positive. This culture was maintained separately and was designated NAB II. EA- and VCA-negative cultures were designated NAB I. Cells of NAB I and II cultures grew as clusters in the medium, which is typical of other lymphoblastoid cell lines. At various intervals after establishment in culture, NAB I and NAB II cultures were analyzed for morphological and antigenic characteristics (Table 1). At the ultrastructural level, most cells in both NAB I and NAB II cultures were immature, lymphoid cells.

Thick Maraglas sections of NAB I cultures (Fig. 1) contained a relatively uniform population of lymphoid cells. Mitotic figures were frequently observed. The large nucleus contained a prominent nucleolus and was bordered by a small basophilic rim of cytoplasm (Fig. 3). Part of the chromatin was marginalized adjacent to the nuclear membrane, but most of the chromatin was dispersed in the nucleoplasm. The cytoplasm contained clusters of free ribosomes, occasional short segments of organized endoplasmic reticulum, and a few round, large mitochondria. Golgi apparatus, when present, were single. A few cells contained lipid droplets. A few larger lymphoblastic cells were also present. NAB I cells were immunologically silent for virus, EA, and VCA expression.

NAB II cultures in early passage contained lymphoid cells and a few large cells, some of which were binucleate (Fig. 1).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Biological characterization of NAB cultures</th>
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<tr>
<td><strong>Cell culture</strong></td>
<td><strong>Passage level</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NAB I</td>
<td>Early (1)</td>
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<tr>
<td>NAB II</td>
<td>Early (1)</td>
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<tr>
<td>NAB I</td>
<td>Mid (36)</td>
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<tr>
<td>NAB I</td>
<td>Late (60)</td>
</tr>
<tr>
<td>NAB II</td>
<td>Late (94)</td>
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<sup>a</sup> Cultures were received without passage designation. Early (1 to 10), mid (36 to 48), and late (60 to 94) refer to Frederick Cancer Research Center passage level, given in parentheses.

<sup>b</sup> Percentage of replicate 100 cells determined by IFL.

<sup>c</sup> Positive designation refers to relative numbers of herpesvirus particles observed by electron microscopy 1+ (few) to 4+ (many) herpes particles.

<sup>d</sup> With IUdR treatment no virus particles were observed, but a herpes-like CPE was seen.

<sup>e</sup> CPE: cytopathological effect; ND, not determined.
2). Ultrastructurally, NAB II cells exhibited lymphoid characteristics and alterations typical of herpesvirus infection (Figs. 4 to 8). All stages of cytopathology of herpes-infected cells were demonstrated. Early cellular alterations included the presence of numerous annulate lamellae (Fig. 8), increased amorphous densities within the endoplasmic reticulum, slight reduplication of nuclear membrane, and pooling of cytoplasmic organelles. This was followed by displacement and condensation of the chromatin at the nuclear membrane, which ultimately led to a distortion and fragmentation of the nucleus. In addition, long stretches of reduplicated nuclear membranes folded many times upon themselves were found (Fig. 5). Bundles of fibrils in the cytoplasm were often observed associated with virus particles (Fig. 6, arrow). Primarily naked herpes particles at various stages of nucleoid maturation were observed within the nuclei of these cells (Fig. 4, double arrows and insets A and B). These particles appeared to leave the nucleus by traversing the nuclear pores. Numerous extracellular enveloped herpes particles (Fig. 4, single arrow and inset C) were observed. These mature herpes particles were highly cell associated and were not observed in negatively stained, concentrated (100- to 1000-fold) supernatant fluids. Occasionally, naked herpes virions were observed within the nuclei of necrotic cells (Fig. 7).

Both NAB I and II cultures contained a higher percentage of viable cells than did other EBV-producing cell lines (P3HR-1, B-95-8) examined in this laboratory. All passage levels of NAB I and II cultures were EBNA positive (Table 1). Concentrated (100- to 1000-fold) supernatant fluids did not induce EBNA in human cord leukocytes or EA in Raji cells. Both NAB cultures superinfected with P3HR-1 virus contained more IFL-positive cells than did the control cultures. NAB II cultures showed a greater increase in immunofluorescent staining than did NAB I cultures (Table 1). However, NAB cultures were not superinfected with B-95-8 virus. NAB I and NAB II cultures exposed to herpes simplex virus 1 or 2 for 4 hr after adsorption did not demonstrate any alteration in their EBV-specific IFL. With continued passage NAB II cultures continued to express low levels of VCA and EA activity (0.5 to 1.0%), but virus particles, as determined by electron microscopy, were not observed. NAB I cultures remained negative for VCA and EA and were never found to contain herpesvirus particles.

NAB I and II cells after 1.5 years in culture displayed gradations in morphological characteristics and different immunological responses to treatment with iUdR. Treatment of NAB I and NAB II cells with iUdR resulted in increased VCA and EA immunofluorescence in NAB II cultures only (Table 1). Examination by light microscopy of thick Maraglas sections of NAB I cells treated with iUdR (Fig. 9) revealed a homogeneous population of cells, which were larger and less basophilic than control cells (Fig. 1). Both nucleus and cytoplasm were hypertrophied. Typical NAB I cells, similar to those observed in Fig. 1, were also present in other areas examined. At the ultrastructural level, there were 2 types of NAB I cells demonstrated: those that appeared normal, similar to NAB I control cells (Fig. 3), and those that showed a marked nuclear and cytoplasmic hypertrophy (Fig. 10). The chromatins in cells affected by the iUdR treatment was highly dispersed. The cytoplasm contained mostly free ribosomes with only short segments of organized endoplasmic reticulum. Occasionally in these cells, tubuloreticular structures within the endoplasmic reticulum were observed. NAB I control cultures did not display these cellular alterations. NAB II cells were consistently more inducible for VCA and EA activity (Table 1). Light microscopic examination of NAB II induced cells revealed a more pleomorphic profile. In addition to smaller basophilic lymphoid cells, there were larger, more weakly basophilic cells, which contained large nuclei and nucleoli. Multinucleated giant cells were also present (Fig. 11). NAB II cultures (Fig. 13) exhibited cellular hypertrophy, early signs of virus infection, nuclear lobulation, and fragmentation with electron microscopic examination. Also present in many cells were tubuloreticular structures (Fig. 12). These tubuloreticular alterations were located primarily in the perinuclear cytoplasm and were pronounced in NAB II cultures. Extensive examination of these cells failed to demonstrate herpesvirus particles. Control cultures at the same passage level did not reveal these cellular alterations.

Cytogenetic analysis of NAB cultures revealed the following: (a) the modal number of chromosomes per NAB I cell was 46 (the range was 43 to 48 chromosomes/cell); (b) in NAB II cells the modal number of chromosomes was 45 (the range was 44 to 47) (Table 2). Only 1 X and no Y chromosomes were found in each cell of both NAB cultures. Fig. 14 shows metaphase spreads typical of NAB I and NAB II cultures and a NAB II karyotype. Eight different marker chromosomes were identified, these were designated M I through M VIII (Fig. 15). Chromosomes 1, 3, 5, 7, 8, 13, 14, and 15 were involved in the formation of these rearrangements. All markers except M VI were seen in both cultures. Marker VI appeared only in NAB II cultures.

Marker I, t(1:5)(p1;q33), was a large submetacentric chromosome, formed by the translocation of the terminal region of the long arm of chromosome 5 onto the deleted short arm of chromosome 1 as the result of a reciprocal translo-

### Table 2

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<th>NAB cytogenetics</th>
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<tr>
<td><strong>No. of cells</strong></td>
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<td>Chromosomes/cell</td>
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<tr>
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<table>
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<th>NAB II</th>
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<tr>
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<td>12 (40)</td>
<td>4 (13)</td>
</tr>
<tr>
<td>VIII</td>
<td>2 (7)</td>
<td>6 (20)</td>
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* Modal chromosome number.

* Numbers in parentheses, percentage.
tion of NAB II cultures by electron microscopy revealed culture and showed many of the behavior patterns typical of cultures derived from patients with Burkitt's lymphoma derived from lymphocytes, and thus they have been described as lymphoid. The ultrastructure of most of the NAB I and NAB II cells was very similar, but NAB II cultures contained a few cells with herpes-like particles. Early cellular alterations observed in NAB II cultures included increased frequency of annulate lamellae and early endoplasmic tubuloreticular alterations. These alterations were noted in many African Burkitt's tumor cells, as well as in cultures of lymphoblastoid cells derived from leukemic and solid tumors (6, 23, 46).

The failure to express EA in the Raji superinfection assay or transforming activity in human cord leukocytes does not make the NAB cultures an in vitro source of an American-derived EBV strain. The passage level of the culture might influence its ability to synthesize virus that is active (18).

NAB cells can be used as another indicator cell line for EA. After superinfection with P3HR-1 virus, the most EA is found in Raji cells, less is found in NAB II cells, and the least is found in NAB I. NAB cultures have some of the characteristics of 2 lines isolated from American Burkitt's lymphoma patients, RA#1 (31) and Su-Am-2 (10). These characteristics are that the lines are EBNA positive, are readily inducible with IUDr, and can be superinfected with P3HR-1 virus.

In IUDR-treated NAB I and II cells, the marked increase in tubuloreticular structures was paralleled by an increase in EA and VCA activity only in NAB II cells. After 5-bromodeoxyuridine treatment of Raji cells, Hampar et al. (19) observed structures in the endoplasmic reticulum that were dependent upon the concentration and duration of the 5-bromodeoxyuridine treatment. The maximal effect was seen 3 days after initiation of treatment, as in our experimental observations. The induction of tubuloreticular structures has been recently reported to be dependent on S-phase treatment (27). These endoplasmic reticulum alterations have also been described in tumor cells of American Burkitt's lymphoma patients (47). There is only 1 report in which a positive correlation between viral antigen as detected by immunofluorescence and the presence of endoplasmic reticulum alterations was obtained (38).

Continued examination of NAB cells treated with IUDR thus far has failed to demonstrate herpes-like virus particles. Similar findings have been reported in both producer P3HR-1 (18) and nonproducer Raji (19) cultures. In these studies, herpes particles were observed by electron microscopy only after removal of the drug.

Karyotypic analysis of NAB cultures revealed the presence of markers I, II, and III in all metaphases analyzed. These chromosomal rearrangements can be used to distinguish NAB cells from other cells in culture, and they confirm that NAB I and NAB II cultures were derived from the same line. The relatively low incidence of markers IV to VIII suggests that they arose in vitro, although their origin in vivo cannot be ruled out. Breakage and reunion at band 14q32 was seen in marker VII, a 1;14 translocation. An 8;14 translocation involving this same band has been reported in 3 other American Burkitt's lymphoma lines (35, 54), and a similar 14q+ rearrangement has been well documented in African Burkitt's lymphoma (28, 33, 54).

It is unlikely that the t(1;14) in NAB was EBV induced, since 14q aberrations have been found in both EBV-positive and EBV-negative lymphomas (54) but never in EBV-positive lymphoblastoid lines (28, 54). Rearrange-
ments involving the 14q3 region also have been reported in other lymphoid disorders (16, 21, 34, 45, 53). The demonstration of a 14q3 aberration in the NAB line indicates that liability of the 14q3 region may be an inherent feature of some lymphoid diseases and that such aberrations may predispose lymphoid cells to abnormal growth, as proposed by McCaw et al. (36).

ACKNOWLEDGMENTS

The authors wish to acknowledge R. D. Bradley for photographic support, M. Gregg for the thick sections, H. Rager for technical assistance in conducting the immunofluorescent assays, and A. Huter for her excellent secretarial assistance. Appreciation is also expressed to Dr. G. Pearson (Mayo Clinic, Rochester, Minn.) for discussion in coordination of this program.

REFERENCES

Biological Characterization of EBNA-positive NAB Cultures


Fig. 1. NAB I culture, early passage, shows characteristics of lymphoid cells, including a prominent nucleus with a small basophilic rim of cytoplasm. × 500.

Fig. 2. NAB II culture, early passage, demonstrates larger lymphoblastoid cells. × 500.

Fig. 3. Electron micrograph of typical NAB I cell silent for virus expression. Characteristics of this cell are hypertrophied nucleolus, ribosome-rich cytoplasm, and scant endoplasmic reticulum. × 15,000.
Fig. 4. Survey electron micrograph of NAB II cell demonstrating typical cytopathological effect of herpes infection: unencapsulated herpes particles (double arrow) and an extracellular encapsulated herpes particle (single arrow). $\times$ 19,200. Inset A, primarily empty herpes capsids, with 1 particle (arrow) containing a ring of nuclear material. $\times$ 132,000. Inset B, unencapsulated herpes virion containing a dense central nucleoid. $\times$ 132,000. Inset C, mature herpes virion enveloped by an additional outer membrane. $\times$ 132,000.
Fig. 5. Part of the nucleus of an NAB II cell demonstrating dense fragmented chromatin, reduplication of nuclear membrane, and immature herpes virions. $\times$ 28,000.

Fig. 6. Portion of a NAB II cell showing bundles of thin fibrils associated with virus particles (arrow). $\times$ 36,000.

Fig. 7. Numerous herpes-type cores and nucleocapsids trapped in cellular debris. $\times$ 51,000.
Fig. 8. Many annulate lamellae are observed in the juxtanuclear cytoplasm of NAB II cells. × 37,400.
Fig. 9. NAB I, late passage, treated with iUdR, demonstrating larger, weakly basophilic cells. × 500.
Fig. 10. NAB I culture, late passage level, treated with iUdR, demonstrating cellular hypertrophy. × 10,500.
Fig. 11. NAB II, late passage, treated with IUdR, demonstrating marked variation in cellular morphology. × 500.
Fig. 12. NAB II cell treated with IUdR showing the typical appearance of perinuclear tubuloreticular arrays. × 28,800.
Fig. 13. NAB II culture, late passage level, treated with IUdR, showing early nuclear fragmentation and endoplasmic reticulum alterations. × 10,500.
Fig. 14. G-banded chromosomes from NAB cells. a, metaphase from NAB I cell with marker chromosomes I, II, III, IV, and V; b, metaphase from NAB II cell, with marker chromosomes I, II, III, IV, V, VII, and VIII; c, karyotype of the cell shown in b.
Fig. 15. Demonstration of the origin of the 8 marker chromosomes of the NAB cell line by comparison with normal chromosomes from NAB cells.
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