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Comparison between growth characteristics of an Epstein–Barr virus (EBV)-genome-negative lymphoma line and its EBV-converted subline *in vitro*

(temperature dependence/suspension cultures/human lymphoid cells/selection)

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ABSTRACT **The GC-BJAB cell line, which carries the Epstein–Barr virus (EBV), was derived from an EBV-genome-negative lymphoma line (BJAB) by EBV infection *in vitro* [G. B. Clements, G. Klein, and S. Povey (1975) *Int. J. Cancer*, in press]. Both lines grow at a similar rate at 37° but they differ at other temperatures. BJAB grows well at 34°, 37°, and 39°. GC-BJAB grows at 37° and 39°, but grows poorly at 34°. At 37°, GC-BJAB cultures can be maintained at the viable state for a long time after having reached saturation density (approximately 10^6 cells per ml). In contrast, BJAB cultures die very soon after having attained similar maximum density. Since the identity of the two cell lines has been critically established [Clements *et al.*; E. Svedmyr and M. Jondal (1975) *Proc. Nat. Acad. Sci USA* 72, 1622–1626; G. Klein, to be published; J. Zeuthen, personal communication] the remarkable differences in their growth properties must be attributed to the EBV genome.**

BJAB is a B-type human lymphoma line, derived from an exceptional African Burkitt lymphoma case in which the cells were negative for DNA of Epstein–Barr virus (EBV) (1). The cells carry surface-bound immunoglobulin, EBV receptor (2), but do not harbor any detectable EBV DNA genome (3) or EBV-determined nuclear antigen (EBNA) (4). By infecting this cell line with transforming EBV derived from the B95-8 strain, a permanently EBV-converted subline was established (5). Designated as GC-BJAB, it is EBNA-positive and harbors one to two EBV genome copies per cell (M. Andersson, personal communication). BJAB and GC-BJAB have the same karyotype (J. Zeuthen and G. Clements, to be published). They resemble each other closely with regard to isozymes, surface immunoglobulin, superinfectability with early antigen (EA) inducing P3HR1-EBV (5), and have identical susceptibility to the lymphocytotoxicity reaction described by Svedmyr and Jondal (6). Presence of the EBV genome and its EBNA product is the only known difference between the two cell lines. Therefore, any biological differences are likely to depend on the viral infection of GC-BJAB. EBV-genome-carrying lymphoid cells are known to possess a remarkable growth advantage in culture. Whereas, EBV-positive Burkitt lymphomas can be established relatively easily as continuous lines, EBV-genome-negative lines are only established with great difficulty (7). EBV-carrying, continuous lymphoid lines can be readily established from the peripheral blood or lymph nodes of seropositive individuals (8, 9). The establishment of continuous cell lines from, e.g., EBV-negative myelomas, lymphomas, leukemias, and nasopharyngeal carcinomas (7) is greatly hampered by the ready outgrowth of contaminating, EBV-genome-positive lymphoblastoid cells. Upon *in vitro* infection of normal B-lymphocytes that have a limited life span, EBV "immortalizes" them, i.e., converts them into permanently EBV-carrying lymphoblastoid lines. All this indicates that the presence of the viral genome confers a considerable selective advantage on lymphoid cells under *in vitro* conditions. It is difficult to study the nature of this advantage by comparing unrelated lines, since there are many differences between them, in addition to presence or absence of the viral genome. The existence of a genome-negative and a genome-positive cell line derived from the same original lymphoma provides a nearly ideal model for such studies. The present paper is a first approach to this problem and demonstrates a remarkable difference in temperature dependence and survival under saturation conditions between the two sublines.

Abbreviations: EBV, Epstein–Barr virus; EBNA, EBV-determined nuclear antigen; FCS, fetal calf serum.

MATERIALS AND METHODS

Cell Passage. BJAB and GC-BJAB cells were routinely passaged in stationary culture at 37°, in an atmosphere of 5% CO_2 in air and 80–90% relative humidity. RPMI 1640 (Gibco) containing 10% fetal calf serum (FCS) was used throughout as growth medium. The medium was supplemented with penicillin, streptomycin, Fungizone, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes).

Incubation Conditions. Cells were incubated in plastic, sterile tubes (Falcon no. 2057), or in tissue culture flasks (Falcon no. 3012) as indicated in each experiment.

Cell Counting. At each time point the number of cells and percent viability were assessed with trypan blue.

EBNA Assay. EBNA was assayed by anticomplement immunofluorescence as described by Reedman and Klein (10). EBNA tests were performed on BJAB, GC-BJAB, and mixtures of the two cells. Before testing, the cultures were put on a Ficoll–Isopaque gradient (11) to remove dead cells. Smears were prepared and fixed by acetone–methanol as described. The Hyland Laboratories goat antiserum to human β_1C/β_1A reagent was used to stain EBNA at a dilution of 1:40. The smears were finally stained with Evans blue (100 mg/liter) for 10 min.

RESULTS

BJAB and GC-BJAB cells have approximately the same doubling time of 17 hr when they grow at 37°, in the cell densi-

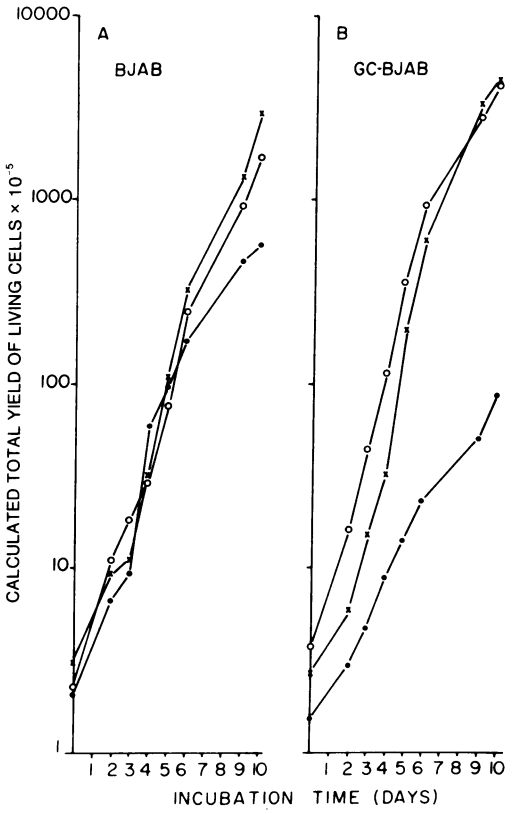

FIG. 1. Growth of (A) BJAB and (B) GC-BJAB in RPMI with different concentrations of FCS. Cells of the two lines were incubated in 10 ml of medium, in tissue culture flasks at 37°, 5% CO_2. At each time point viable cells were counted with trypan blue. After each counting, cultures were diluted with fresh medium to adjust the cell number to 1 to 2 × 10^5/ml. The culture volume was kept constant (10 ml). The calculation of the total living cell yield is based on the number of living cells counted, and on the dilutions performed with each culture. ●, RPMI with 1% FCS; ○, RPMI with 5% FCS; ×, RPMI with 10% FCS.

ty range between 10^5 and 5 × 10^5 cells per ml. Fig. 1 (A and B) shows the growth of the two cell lines in media with various concentrations of FCS. The cultures were diluted every day with fresh medium to a final cell density of 1 to 2 × 10^5/ml. There was no clear difference in the growth of the

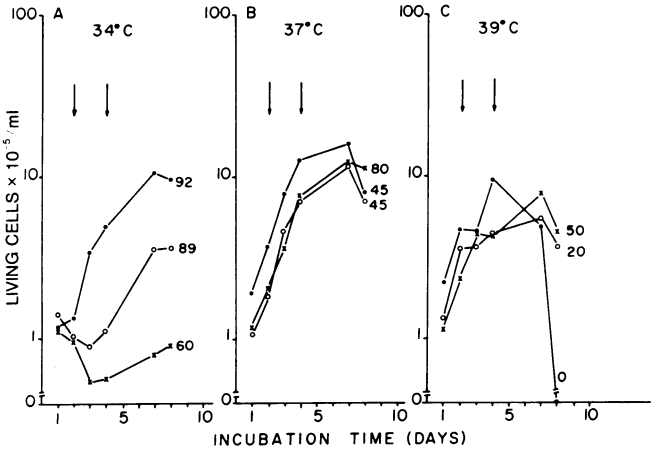

FIG. 3. Growth of BJAB and GC-BJAB at different temperatures. Cells (2 × 10^4) of each line were incubated in 2 ml of RPMI with 10% FCS in tubes, at different temperatures, with 5% CO_2. Mixtures of both (10^4 each) were also incubated under the same conditions. The medium was changed twice, as indicated by the arrows. The numbers at the end of each curve correspond to the percentage of living cells at the end of the incubation time. ●, BJAB; ×, GC-BJAB; ○, BJAB + GC-BJAB.

two cells at 5% and 10% FCS in RPMI. The growth of both lines was reduced in media with 1% FCS.

In another experiment, shown in Fig. 2, 2 × 10^4 cells of the two lines were allowed to grow at 37° in RPMI with different FCS concentrations, without any medium change. They reached a similar maximum density after approximately the same time, 6–7 days. Until this point, viability was >90% in both lines (results not shown). The subsequent course was very different. In the BJAB culture, a steep fall of viability occurred between day 8 and 10 and the culture was dead by day 14. GC-BJAB survived with a relatively steady number of living cells until day 20–30, i.e., at least 20 days after maximal cell density was reached. The difference between the two cell lines was evident in four pairs of cultures, compared at different concentrations of FCS.

Fig. 3 shows the growth curve of 2 × 10^4 BJAB and GC-BJAB cells and a 1:1 mixture, incubated at three different temperatures. The GC-BJAB did not grow or grew poorly at 34°, in contrast to BJAB that grew at a virtually equal rate at 34°, 37°, and 39°. At 39°, BJAB showed the same abrupt

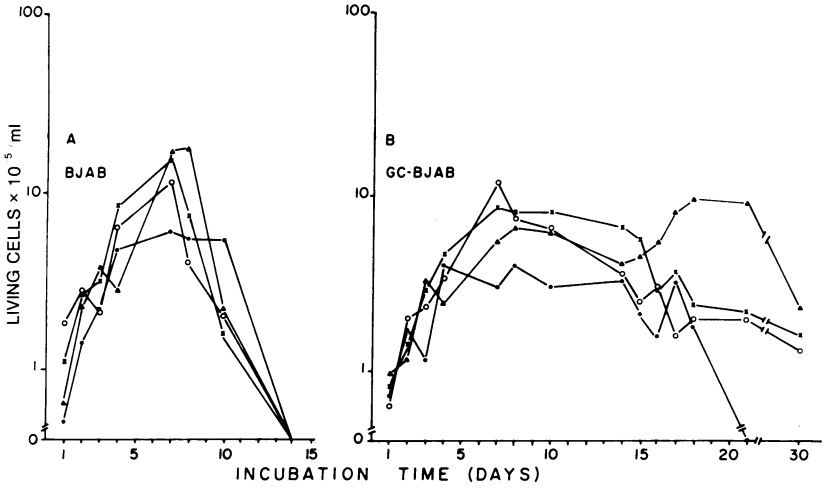

FIG. 2. Growth of (A) BJAB and (B) GC-BJAB without refeeding in PRMI with different concentrations of FCS. Cells (2 × 10^4) of each line were cultured in 2 ml of medium at 37°, 5% CO_2. Viable cells were counted at each time point. ●, RPMI with 1% FCS; ○, RPMI with 5% FCS; ×, RPMI with 10% FCS; ▲, RPMI with 20% FCS.

Table 1. Relative frequency of EBNA-positive and EBNA-negative cells

Initial cell culture	Incubation temperature		
	34°	37°	39°
BJAB	All negative	All negative	Not done
GC-BJAB	All negative	All positive	All positive
BJAB and GC-BJAB (1:1 mixture)	684 negative and 177 positive*	All positive	All positive

Measurements were made eight days after single or 1:1 mixed incubation of BJAB and GC-BJAB. For experimental details, see legend to Fig. 3.

* The positive cells appeared small, and in poor condition.

fall in viability shortly after the peak density was reached as in the experiment depicted in Fig. 2. Also in line with this experiment, the viability of GC-BJAB at 39° was not affected after peak density was reached. At 37°, the viability of BJAB started dropping shortly before the experiment was terminated. At 34° there was no drop of viability during the observation period.

The two cell lines were also mixed (1:1) and incubated at three different temperatures. At the end of the experiment (8 days) EBNA-negative cells were differentially counted on stained smears (Table 1). At 34° the mixture contained 79% EBNA-negative cells; 21% were EBNA-positive but they were small and in poor condition. At 37° and 39° the cells were all EBNA-positive, however. These results indicate that even in mixtures of the two lines, BJAB cells grow preferentially at 34° while GC-BJAB remains stationary and degenerates. At 37° and 39°, BJAB cells perish at saturation density whereas GC-BJAB survive, whether alone or in mixture.

DISCUSSION

The results show that the BJAB and its EBV-converted GC-BJAB subline differ in two important respects. BJAB grows well at 34°, 37°, and 39° while GC-BJAB grows at 37° and 39°, but not, or just poorly, at 34°. At 37° both lines have similar doubling times, and reach a similar saturation density. BJAB is sensitive to saturation conditions and dies rapidly after having reached maximal cell density, unless diluted with fresh medium. In contrast, GC-BJAB cells can survive at saturation density for a surprisingly long time.

Since the presence of the EBV-genome in the GC-BJAB line is the only known difference between the two lines, it seems that the viral genome or its products have brought about two changes: dependence on some limiting factor or condition that cannot be made at 34°, and increased resistance to saturation conditions. Whether both changes are due to the pleiotropic effect of the same viral gene or are independent is an interesting question. As a next step, it will be informative to compare other, independently EBV-converted sublines of BJAB. Also, we have recently succeeded in EBV-converting yet another genome-negative lymphoma line, Ra1 (G. Klein, B. Giovanella, A. Westman, J. Stehlin, and D. Mumford, to be published). Since the latter conversion was achieved with a different EBV strain, the regularity of the EBV-induced changes can be studied in comparison with the BJAB-GC-BJAB pair.

Our findings also raise the question whether the differences demonstrated in this study can be used for the selective establishment of EBV-positive and negative lines, respectively. If so, cultivation at 34° may favor the establishment of genome-negative lymphoma lines, in competition with contaminating genome-positive cells. Conversely, cultivation under saturation conditons may facilitate the emergence of EBV-converted cell lines from *in vitro* EBV-infected lymphoma lines and perhaps also the outgrowth of *in vitro* EBV-immortalized normal lymphocytes.

Note Added in Proof. After this paper had been submitted for publication experiments were also carried out with the EBV-negative American Burkitt line, Ra1, and four of its recently EBV-converted, EBNA-positive sublines. All four EBV-carrying sublines showed a similar prolonged survival after reaching maximal density as the GC-BJAB-line in the present study. In contrast, the EBNA-negative Ra1-line died rapidly after reaching maximal cell density, similar to the EBV-negative BJAB-line.

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