Can Epstein–Barr Virus Infect and Transform All the B-Lymphocytes of Human Cord Blood?

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SUMMARY

Quantitative aspects of Epstein–Barr virus infection and transformation of human neonatal B-lymphocytes have been investigated. 72 to 90% B-cells were obtained with enrichment. Of the B-cells, 19 to 97% showed nuclear antigen (EBNA) 2 days after infection. A difference between different B-cell donors in susceptibility to infection was noted. Analysis of the virus dose–response curves obtained with twofold virus dilutions showed that one virus particle is sufficient to induce EBNA in a cell. Of the infected cells, 50 to 95% multiplied in microtitre wells containing a human fibroblast feeder layer, while only a small proportion established growing colonies in soft agarose, that could be picked up and subcultured.

INTRODUCTION

Human and some primate B-lymphocytes are the only known target cells for Epstein–Barr virus (EBV) infection in vitro (Einhorn et al., 1978; Henderson et al., 1977). Katsuki et al. (1977) and Steele et al. (1977) have shown that it is most likely the IgM-bearing B-lymphocytes that are the target cells in human umbilical cord blood while IgM-, IgG- and IgA-bearing B-lymphocytes may be infected in adult peripheral blood (Steele et al., 1977). The infection leads to the establishment of polyclonal, continuously growing B-lymphoblastoid cell lines (Chang & Golden, 1971; Gerber et al., 1969; Henle et al., 1967; Miller et al., 1971; Pope et al., 1968; Schneider & zur Hausen, 1975). Several assays have previously been utilized to follow and quantify EBV-directed transformation (immortalization): stimulation of DNA synthesis, outgrowth of cells, growth on feeder layer and growth in soft agarose after infection (Chang et al., 1976; Henderson et al., 1977; Katsuki & Hinuma, 1976; Mizuno et al., 1976; Robinson & Miller, 1975; Sugden & Mark, 1977; Yamamoto & Hinuma, 1976). In such studies, between 0.1 and 10% of the target cells was estimated to be transformed. Henderson et al. (1977) and Sugden & Mark (1977) found that the transformation of target cells followed a linear response with dilution of the virus, and this response could most easily be aligned with a one-hit curve, indicating that one virus particle is sufficient to transform a cell.

The purpose of our study was to relate the infectibility of human cord blood B-lymphocytes as judged by nuclear antigen (EBNA) induction to the efficiency of growth of freshly infected cells in two systems: in suspension and in a semi-solid medium. The EBV was concentrated to obtain excess virus for the estimation of the number of infectible cells. The target B-lymphocytes were purified to allow direct deduction of the number of infectible and growing cells after infection.

METHODS

Fractionation of cells. Heparinized human cord blood cells were collected, and lymphocytes were separated by centrifugation on Ficoll–Isopaque (Böyum, 1968). Lymphoid cells at the interphase of the gradient were collected, washed with medium (RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum) and macrophages removed by carbonyl iron treatment for 30 min at 37 °C according to Jondal (1974). B-cells were first enriched by passage over a nylon wool column (Cornain et al., 1975). Cells (10 x 10⁶) were incubated for 1 h at 37 °C in 5 ml plastic syringes each containing 0-425 g of nylon wool, pretreated and washed with 20 ml of warm tissue culture fluid.
medium. Unabsorbed cells were washed out with warm culture medium; adherent cells (B-enriched cell population) were removed by incubating the nylon wool in 15 ml of warm foetal calf serum for 10 min at room temperature. The cells were then washed and resuspended in culture medium. A suspension of 2.5% washed sheep erythrocytes was added at a ratio of one part to five parts of cells, the mixture was layered on a Ficoll–Isopaque gradient and centrifuged for 20 min at 2000 rev/min. The interphase was collected and the percentage of B-cells was estimated.

**Surface markers.** The proportion of the cells possessing surface immunoglobulin was determined by staining with fluorescein isothiocyanate (FITC)-labelled polyvalent anti-human immunoglobulins. Fluorescence-positive and -negative cells were counted in a Leitz Orthoplan fluorescence microscope at × 640; 100 to 200 cells were counted in each sample.

**Preparation of EBV and infection.** An EBV-producing marmoset cell line B95-8 (Miller & Lipman, 1973) was cultured in RPMI 1640 medium supplemented with 10% foetal calf serum, 100 μg/ml streptomycin and 100 units/ml of penicillin in a humid incubator at 37 °C with 5% CO₂. One-week-old culture medium was collected and the cells were removed by centrifugation at 3000 rev/min for 30 min. The supernatant was collected and centrifuged at 10000 g for 120 min and the pellet was resuspended in 0.15 M phosphate-buffered saline pH 7.4 to provide 50-fold concentrated virus. The virus concentrate was passed through a Millipore filter with 0.45 μm pore size and stored at 4 °C for no longer than 15 days. During this period no decrease in the infectious activity of the virus preparation was noticeable.

One ml of appropriate virus dilution was used to infect replicate tubes each with 10⁶ cells. The mixture was incubated with the cells for 1 h at 37 °C. After virus adsorption, the cells were centrifuged for 10 min at 2000 rev/min. The supernatant was then removed and the cells resuspended in 1 ml culture medium.

Forty-eight h after infection, the cells were tested for EBV-associated nuclear antigen (EBNA), and processed for the growth assay in micro-wells and the clonal transforming assay in soft agar.

**Immunofluorescence.** Cell smears were prepared and stained for EBNA according to Reedman & Klein (1973), by the acicomplementary immunofluorescence (ACIF) method. At least 500 cells were counted in each sample, differentiating between fluorescence-positive and -negative cells. A Leitz Orthoplan fluorescence microscope was used at × 640. The staining also labels complement receptors (Bianco et al., 1970; Einhorn et al., 1978; Yefenof et al., 1976), which are present on all or most of the B-cells. Immunofluorescent labelling of complement receptor differs both in location and morphology from that due to EBNA. We checked the reproducibility of the counting of the EBNA smears by re-counting the same slide several times, and the standard deviation of the counting error was ± 4.5% at high EBNA induction and ± 1.4% at low induction.

**Preparation of feeder layer.** Subcultured human foetal lung fibroblasts (after approximately 10 passages) were collected in RPMI 1640 with 10% heat-inactivated foetal calf serum. A 0.2 ml amount of an appropriate cell suspension was plated in flat-bottom micro-wells (Falcon, Microtest II) and 2 ml in Petri dishes (35 mm, Falcon Plastics). Fibroblasts were allowed to grow as monolayers, and when saturation density was reached the cells were irradiated with 6000 rad (Siemens roentgen unit, 220 kV, 15 mA, 1 mm Al-filter).

**Growth assay in micro-wells by limiting cell dilution.** The B-enriched cell populations were infected with undiluted B95-8 virus concentrate and incubated in plastic tubes (17 × 100 mm, Falcon) for 48 h at 37 °C in a humidified CO₂-enriched atmosphere. Infected cells diluted in warm medium to 100, 10 or 1 cell per 0.2 ml were then added to fibroblast feeder layers in micro-wells. An identical volume of the infected and appropriately diluted cell suspension was added. Half of the medium in each well was carefully removed every 5 days, and fresh medium added. The plates with infected cells were kept for at least 15 days at 37 °C in humidified air in a CO₂ incubator, and each well was screened under an inverted microscope for cell growth. In each experiment uninfected B-cells were included as control cultures.

**Clonal growth assay in soft agarose.** Confluent feeder layers of human lung fibroblasts were grown in Petri dishes and irradiated, and 1 ml 0.45% agarose medium was added at 40 °C (Sea Plaque agarose: Biochemical Division, Maine Colloid Corp.). Subsequently, the dishes were allowed to stand for 2 h at room temperature and then for 24 h in the humidified CO₂ incubator at 37 °C. B-enriched cells (2.5 × 10⁴) infected with twofold dilutions of concentrated B95-8 virus were suspended in 1 ml of 0.35% agarose medium and poured onto the first layer of agarose. The cell cultures were kept for 1 week at 37 °C in a humidified CO₂ incubator. The number of colonies growing in agarose was counted under an inverted microscope at low magnification. This number is expressed as the mean of three plates for each virus dilution. A colony was considered positive when it contained at least 25 cells.

**Cloning of cells.** Single clones were picked with silicone-treated capillaries under an inverted microscope. The isolated clones were then grown in 0.4 ml of medium (RPMI 1640 + 20% heat-inactivated foetal calf serum) in plastic tubes (12 × 75 mm, Falcon) and incubated in a humidified CO₂ incubator at 37 °C. The cultured clones were re-fed every 5 days after gently removing half of the supernatant. Growing clones were transferred after 15 days to 17 × 100 mm plastic tubes and fed with 1 ml culture medium. Subsequently, they were transferred to 25 cm² tissue culture flasks (Falcon) at a cell concentration of 3 × 10⁵ cells/ml.
EBV infection of human peripheral B-cells

Table 1. Frequency of B-cells, EBNA-positive cells and calculated percentage of EBNA-positive cells from the B-cell population in different experiments

<table>
<thead>
<tr>
<th>Virus preparation</th>
<th>Donor of lymphocytes</th>
<th>% B-cells</th>
<th>% EBNA-positive cells</th>
<th>% EBNA-positive cells from the B cells (calculated)</th>
<th>Symbol of curve in Fig. 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>I</td>
<td>90</td>
<td>61</td>
<td>67</td>
<td>△</td>
</tr>
<tr>
<td>B</td>
<td>II</td>
<td>83</td>
<td>56</td>
<td>79</td>
<td>○</td>
</tr>
<tr>
<td>C</td>
<td>III</td>
<td>80</td>
<td>63</td>
<td>79</td>
<td>○</td>
</tr>
<tr>
<td>C</td>
<td>IV</td>
<td>72</td>
<td>13</td>
<td>19</td>
<td>▽</td>
</tr>
<tr>
<td>D</td>
<td>IV</td>
<td>72</td>
<td>24</td>
<td>33</td>
<td>▽</td>
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<td>D</td>
<td>V</td>
<td>90</td>
<td>55</td>
<td>63</td>
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<tr>
<td>E</td>
<td>V</td>
<td>90</td>
<td>87</td>
<td>97</td>
<td>▽</td>
</tr>
<tr>
<td>F</td>
<td>VI</td>
<td>78</td>
<td>53</td>
<td>68</td>
<td>▽</td>
</tr>
<tr>
<td>G</td>
<td>VII</td>
<td>72</td>
<td>25</td>
<td>35</td>
<td>▽</td>
</tr>
<tr>
<td>H</td>
<td>VIII</td>
<td>82</td>
<td>27</td>
<td>32</td>
<td>▽</td>
</tr>
</tbody>
</table>

RESULTS

Response of enriched B-lymphocytes to EBV infection

B-lymphocytes from cord blood from eight different donors were tested for EBNA induction after infection with active, concentrated B95-8 virus. The percentage of B-cells varied between 72 and 90% (Table 1), as estimated by surface immunoglobulin staining with FITC-conjugated anti-human immunoglobulin antibodies. The proportion of EBNA-positive cells among the B-cells varied between 19-4 and 96-6% (arithmetic mean: 56.5%).

After twofold dilutions of the virus, titration curves were obtained as shown in Fig. 1. At high virus concentrations there was little or no decrease of the EBNA induction response, while this dropped rapidly with further dilution of the virus.

Theoretically, it is possible to deduce the minimum number of virus particles required to infect a cell from the slope of the titration curves. In Fig. 2, two experiments are shown, where the lymphocytes were infected in triplicates with different twofold virus dilutions. The titration curves are compared with theoretical one- and two-hit kinetic curves, calculated by adopting the analysis of the number of virus particles required to induce a plaque in lytic virus-host cell systems (Davis et al., 1968). One EBNA-positive cell then corresponds to one infectious unit of the virus preparation, like one virus-induced plaque would be in a virus-plaque assay. This assumption is valid, since the EBNA-positive cells have not yet started to proliferate at the time of harvest. Another prerequisite for the comparison is that the target cells are in excess compared to the number of infectious units, and thus the analysis is only valid for lower virus concentrations. The theoretical one-hit curve includes all virus dilution points, but the two-hit or multi-hit (not shown) curves include only three or fewer points. This indicates that one virus particle is sufficient to induce EBNA in a target B-lymphocyte. A similar analysis has been performed on the titration curves in Fig. 1 at the lower virus concentrations, but the theoretical hit curves are not shown in the figure. All these five titrations also show a dose-response compatible with the hypothesis of one-hit kinetics.

In two experiments the same virus preparation was tested on two different donors (Table 1 and Fig. 1). A difference in EBNA induction was noted between the donors (19 to 79%; 33 to 63%).

Ability of infected cells to grow in suspension

Infected B-cells were seeded on a feeder layer of irradiated human foetal lung fibroblasts in microplates. The microwells were prepared with 100, 10 or 1 cell(s)/well. After 15 days, the wells were surveyed for growth. The cells were observed as clumps or flakes composed of round cells, formed with an increase in cell number (Fig. 3a, b). The results are summarized in Table 2. The number of wells showing growth is related to the number of EBNA-positive cells. In Table 2, the expected number of growing cells was calculated according to the Poisson distribution of EBNA-positive cells in the wells. There was good agreement between the expected and the
Fig. 1. Percentage of EBNA-positive cells as a function of virus dilution. B-lymphocytes enriched from human umbilical cord blood were infected with twofold dilutions of concentrated B95-8 virus. These titrations were performed in five experiments from which data are also included in Table 1, where the symbols of this figure are provided. Two pairs of curves originate from experiments with the same donor, but with different virus batches (○ and ▼).

Fig. 2. Percentage of EBNA-positive cells as a function of virus dilution at lower multiplicities of infection. Virus titration on two different donors is shown. The plotted results are the means of triplicate cultures for each virus dilution. SD is indicated by vertical bars. Theoretical virus-hit kinetics curves were calculated by adopting the analysis of one-hit (—), two-hit (— — ) and multi-hit (not shown) kinetics for virus titration by the plaque method (Davis et al., 1968). For this purpose, one EBNA-positive is considered equivalent to one virus-induced plaque, i.e. both are the result of infection with one infectious unit of the virus preparation (as the EBNA-positive cells have not yet started growing at the time of analysis).

Observed number of wells with growth: the observed number was slightly lower than the expected number. The results imply that more than 60% of the EBNA-positive cells are able to grow under these conditions.

Cells picked up and pooled from wells seeded with 10 or 100 cells could be propagated in suspension culture. Such cultures contained ≥ 90% EBNA-positive cells. The wells seeded with one cell contained too few cells at the end of the experiment for further subculturing. Wells loaded with uninfected cells or infected cells but no feeder layer showed no persisting growth during the 15 day period.

**Ability of recently infected cells to grow in soft agarose**

A growth assay in soft agarose was adopted to allow comparison of the number of infected cells (EBNA-positive) with cells growing in suspension with the frequency of growth in semi-solid medium. Infected and uninfected cells were plated in soft agarose (0.35%) 2 days after
EBV infection of human peripheral B-cells

Table 2. Growth of EBV-infected cells on feeder layer in micro-well system followed for 15 days

<table>
<thead>
<tr>
<th>% B cells*</th>
<th>% EBNA-positive cells†</th>
<th>Cells/well</th>
<th>Positive/total</th>
<th>Observed</th>
<th>Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>24</td>
<td>100</td>
<td>15/16</td>
<td>93</td>
<td>99·9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>8/16</td>
<td>50</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>3/16</td>
<td>18</td>
<td>21</td>
</tr>
<tr>
<td>83</td>
<td>51</td>
<td>100</td>
<td>8/8</td>
<td>100</td>
<td>99·9</td>
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<tr>
<td></td>
<td></td>
<td>10</td>
<td>12/24</td>
<td>50</td>
<td>99·4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>4/16</td>
<td>25</td>
<td>40</td>
</tr>
<tr>
<td>52</td>
<td>12</td>
<td>100</td>
<td>14/17</td>
<td>82</td>
<td>99·9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>14/33</td>
<td>42·4</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>4/49</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>90</td>
<td>87</td>
<td>100</td>
<td>24/24</td>
<td>100</td>
<td>99·9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>38/48</td>
<td>79</td>
<td>99·9</td>
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<tr>
<td></td>
<td></td>
<td>1</td>
<td>35/64</td>
<td>54</td>
<td>58</td>
</tr>
</tbody>
</table>

* Detected with fluorescent anti-Ig at the start of the infection (time 0).
† Detection with ACIF 48 h after infection.
‡ Calculated from the Poisson distribution, on the assumption that all EBNA-positive cells could grow, but not the EBNA-negative.

infection. One week after plating, large colonies could be observed in plates with infected cells (Fig. 4), while no cells were seen in those seeded with uninfected cells.

Fig. 5 shows the number of clones initiated by different virus dilutions, counted on day 7 after plating (day 9 after infection). The number of colonies shows a linear relationship to the virus dose at low concentrations, while there is no such relationship at higher virus concentrations.

Between 1·1 and 1·4% of the total number of plated cells developed into colonies. Assuming that only EBNA-positive cells were able to grow in the agarose, 1·4 to 3·7% of these cells grew into colonies. This assumption is supported by the fact that it was possible to establish picked colonies as continuously growing suspension cultures, and these cultures showed close to 100% EBNA-positive cells.
Fig. 4. One colony of infected lymphocytes in soft agarose 9 days after infection of B-lymphocytes with B95-8 virus. Unstained, living cells photographed through an inverted microscope (Diavert, Leitz, × 320).

Fig. 5. Number of colonies developing in soft agarose as a function of twofold virus dilutions of concentrated B95-8 virus. 2.5 × 10^4 enriched B-lymphocytes from human cord blood were added to each plate [82% (●), 78% (○) and 61% (▲) B-lymphocytes]. The mean ± sd of triplicate plates is shown.

**DISCUSSION**

EBNA induction after B-lymphocyte infection was registered on day 2 after infection. This time point was chosen for optimal induction of EBNA prior to the onset of growth stimulation. EBNA appears 10 to 25 h after the infection of lymphocytes, followed by the stimulation of cellular DNA synthesis at 40 h (Aya & Osato, 1974; Einhorn & Ernberg, 1978; Menezes et al., 1976), mitosis at 48 h (I. Ernberg, unpublished observation) and, subsequently, the first cell division. Thus, the frequency of EBNA-positive cells on day 2 reflects the number of initially infected cells before their number is changed by cell division.

Infection with different virus dilutions showed a plateau of EBNA induction at high virus concentrations. This implies that all susceptible cells in the target population were infected. Between 13 and 87% of the whole lymphocyte population was infected. Only B-lymphocytes are susceptible to EBV-infection (Einhorn et al., 1978; Jondal & Klein, 1973). Thus, taking into account the small variable contamination of non-B-lymphocytes, between 19 and 97% of the B-cells were infected. In six out of ten experiments, more than half of the B-cells were infected. Considering that almost all susceptible cells were infected, and that there was no influence on the number of infected cells due to cell proliferation, we conclude that usually the majority of the B-cells in human cord blood may be infected. Moss et al. (1981) have reported a similar situation with adult B-lymphocytes. When they studied the appearance of EBV-associated cell surface antigen (LYDMA) in relation to the appearance of EBNA after infection of adult human B-lymphocytes, they found a high level of EBNA induction. In experiments where they blocked B-cell proliferation with 10 μM-thymidine, they still detected 70 to 76% EBNA-positive cells after infection.

We cannot exclude the possibility that there may be a resistant subclass of B-lymphocytes. Actually, Tsukuda et al. (1982) have recently demonstrated, in this laboratory, that by transplanting EBV receptors to purified B-lymphocytes, they could enhance virus-induced
EBV infection of human peripheral B-cells

Table 3. Summary of infectibility and growth in micro-wells and agarose, from ten different experiments

<table>
<thead>
<tr>
<th>Percentage</th>
<th>Frequency of B-cells</th>
<th>Frequency of EBNA-positive cells</th>
<th>Frequency of EBNA-positive cells/B-cells</th>
<th>Frequency of growth on feeder layer/EBNA-positive cells</th>
<th>Frequency of growth in agarose/EBNA-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72-90</td>
<td>13-87</td>
<td>19-97</td>
<td>50-95</td>
<td>1.4-3.7</td>
</tr>
</tbody>
</table>

immunoglobulin synthesis in these cells after EBV infection. The receptors were transferred by membrane vesicles from EBV receptor-positive cell lines. Moreover, they observed a relatively larger proportion of IgG-producing cells, compared to IgM-producing cells, in the receptor transplanted population. One possible interpretation of this finding is that IgG-bearing B-lymphocytes partly lack, or have a low concentration of, EBV receptors, and thus would be more difficult to infect than IgM-positive B-lymphocytes.

The variation observed as between donors may be ascribed to variable conditions in the target cells, to a difference in susceptibility between B-cell subpopulations, and to variations between virus batches. The latter possibility is unlikely in the experiments where the response reached plateau level with concentrated virus. However, two virus batches tested against the cells of the same donor resulted in different levels of EBNA induction. The difference found in sensitivity to infection between different donors in two experiments is interesting. Unfortunately, material derived from fresh human cord blood does not allow repeated experiments on the lymphocytes from the same donor. Thus, the possibility of different susceptibility of different donors has to be further investigated in purified B cells from adults.

The relation between virus dose and EBNA induction is close to being linear at low virus concentrations. When the target cells are in excess, conclusions may be drawn about the slope of the dose–response curve. If the experimental error is taken into account this curve is most easily aligned with a one-hit response. Thus, one virus particle is enough to induce EBNA in a B-cell. Other authors have shown that virus-induced growth in semi-solid medium and in suspension also follows an ideal one-hit curve on virus titration (Henderson et al., 1977; Sugden & Mark, 1977).

The efficiency of the growth of EBV-infected cells on feeder layers has been reported to be high (Henderson et al., 1977). In this paper we report an outgrowth frequency of 50 to 95% of the EBNA-positive cells in such a system. The efficiency of outgrowth after plating on average one cell/well in micro-plates, is remarkable (63 to 93% of the expected, assuming that all EBNA-positive cells had been growing). From micro-wells with initially 10 or 100 cells plated, the cells could be isolated and further subcultured. We have also managed to subculture cells from wells with one cell originally plated. However, this requires 6 to 8 weeks proliferation in the original micro-plate until the cell number is sufficient. For technical reasons the number of clones isolated in this way is low (I. Ernberg, unpublished results).

Nilsson et al. (1977) have shown that growth in soft agarose is related to tumourigenicity among EBV-carrying cell lines. Colony formation in soft agarose has also been used to monitor EBV-induced transformation (Mizuno et al., 1976; Sugden & Mark, 1977). It was therefore of interest to see whether freshly infected B-lymphocytes could develop clones in agarose, and if so, how this was related to the primary infection of these cells as measured by EBNA induction. The results show that a smaller population of cells, compared with that of the cells growing in suspension, is able to grow in soft agarose: 1.4 to 3.7% of the EBNA-positive cells. The colonies formed could be picked and subcultured. The developing cell lines were EBNA-positive.

The results are summarized in Table 3: a large proportion of cord B-lymphocytes may be infected and a majority of the infected cells start to grow in suspension. However, only a small fraction is able to grow in semi-solid medium.

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