# Epstein-Barr Virus Interactions with Human Lymphocyte Subpopulations: Virus Adsorption, Kinetics of Expression of Epstein-Barr Virus-Associated Nuclear Antigen, and Lymphocyte Transformation

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In order to further understand Epstein-Barr virus (EBV)-lymphocyte interactions, we investigated a chain of events including: (i) EBV binding to human lymphocyte subpopulations; (ii) the earliest appearance of EBV-determined nuclear antigen (EBNA) in the lymphocytes after EBV infection; and (iii) establishment of continuous lymphoblastoid cell lines (LCL) by infecting with EBV different types of lymphocyte preparations from the same as well as from different donors. By using direct membrane immunofluorescence assay, we found that only a small fraction of human peripheral blood and cord blood lymphocytes (CBL), and possibly less than 31% of the T cell-depleted lymphocyte population, carry receptors for P3HR-1 strain of EBV. The number of cells carrying receptors for EBV did not vary considerably among different blood lymphocyte populations from several normal donors. EBV adsorption on lymphocyte subpopulations showed that purified thymus-dependent (T) cells and thymocytes did not adsorb EBV, in contrast to T cell-depleted lymphocyte populations and lymphoid cells from fetal liver and spleen. In CBL infected with EBV strain B95-8, EBNA was detected by anti-complement immunofluorescence as early as 18 h after infection. This indicates that EBNA is the earliest detectable EBV-determined intracellular antigen to appear after infection and before or during lymphocyte transformation by EBV. Transformation was observed only in lymphocyte cultures containing detectable thymus-independent B cells but not in cultures of purified T cells. With one exception (ES-B-1), all the EBV-transformed LCL from different origins carried surface-bound immunoglobulins (a B cell marker). These included also the 10 LCL obtained by infecting cultures of adherent cells from different donors. With regard to its surface markers, ES-B-1 appeared to be an exceptional EBV genome-carrying line, and it also lacked the ability to form spontaneous rosettes with sheep erythrocytes (a T cell marker). Therefore, it is possible that ES-B-1 was derived from an atypical B cell or B cell precursor or from a so-called "null cell" transformed by EBV.

Transformation of human and simian lymphocytes into established lymphoblastoid cell lines (LCL) is a well-known biological property of Epstein-Barr virus (EBV) (4, 6, 14, 19).

Transformation of thymus lymphoid cells by EBV and the establishment of several LCL from these cultures was reported by Pope et al. (19) in 1968. However, when cells of seven of these lines (kindly supplied by J. H. Pope) were examined in our laboratory, it was found that they did not form spontaneous rosettes with sheep erythrocytes (SRBC) and that they had readily detectable amounts of surface-bound immunoglobulins (unpublished data); these two features are characteristic of human thymus-independent B lymphocytes (3, 9). Recently, Pattengale et al. (17) reported that transformation could not be obtained by infecting fetal thymocytes with EBV. Jondal and Klein (8) described evidence for EBV receptors on B lymphocytes by means of a rosetting procedure; however, the percentage of EBV receptor-positive cells could not be determined by this method. In a preceding communication, we reported the appearance of EBV-determined nuclear antigen (EBNA) within 1 to 3 days

postinfection in lymphocytes from fetal spleen, bone marrow, and liver (11); however, no EBNA was detected in infected thymus cells from the same fetus. Recently, others have also suggested that EBV may transform only thymus-independent B cells (24, 27).

The present study was undertaken to investigate the following aspects of EBV-lymphocyte interactions: (i) the proportion (percentage) of human lymphocytes from peripheral blood and umbilical cord blood that bears receptors for EBV as detected by direct immunofluorescence assay of adsorbed virus on fresh lymphocytes; (ii) the earliest time at which EBNA could be detected by an anti-complementary immunofluorescence method (22) (previous observations [11] had suggested that EBNA precedes EBV-induced intracellular antigens, thus possibly being a useful early marker for lymphocyte transformation by EBV); and (iii) to learn whether any LCL, without conventional B cell features, could be obtained by EBV infection of purified lymphocyte subpopulations. Such exceptional lines, if available, would strongly suggest that EBV may also transform cells other than typical B lymphocytes. The results show that a small fraction of blood lymphocytes and not all the B cells carry receptors for P3HR-1 EBV and that EBNA can be detected 18 h after infection, and they suggest that EBV may transform cells other than typical B lymphocytes.

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## MATERIALS AND METHODS

**Cell lines.** All the lines used were grown in medium RPMI 1640 (RPMI) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 $\mu$g/ml) as described (12). The lines used were: (i) EBV producer P3HR-1 (7) and (ii) B95-8 (13) lines used for virus production; (iii) Raji cells (21) used as EBV receptor-positive and EBNA-positive control cells; (iv) BJA-B (14) used as EBNA-negative and surface immunoglobulin-positive control cells; and (v) Molt 4 (15) used as EBNA-negative and surface immunoglobulin-negative control cells (see below).

**Virus preparations.** Since there was no evidence for differences at a serological or antigenic level between EBV strains (2, 12), two strains, one for each type of experiment, were used. Thus, virus adsorption tests were carried out with P3HR-1 EBV, whereas for studies on EBNA and lymphocyte transformation EBV strain B95-8 was used.

B95-8 EBV was prepared, titrated, and stored as described earlier (12). Two B95-8 EBV preparations were used; their lymphocyte-transforming titers were $10^{4.8}$ and $10^{5.4}$ $\log_{10}$ 50% transforming units/ml, respectively. In preliminary assays and in controls

(see Table 4), 0.1 ml of these EBV preparations per $10^6$ umbilical cord blood lymphocytes (CBL) transformed these cells between 6 and 14 days as judged by the criteria defined previously (12).

Two preparations of EBV were used in the adsorption experiments. The first was a 70× concentrated preparation of virus from the supernatant of HRIK cell line culture fluid (10). This HRIK EBV was received from Pfizer, Inc. (prepared by K. A. Traul, within the special Virus Cancer Program of the National Cancer Institute, and kindly arranged by R. Manaker). Preparations from the same virus pool (lot no. 3269-188) stored frozen at −85 C were used for one series of EBV adsorption tests (Table 1). The choice of this Pfizer HRIK EBV for our first set of adsorption assays was based on the fact that it was the most suitable virus lot we had at that time as judged by the results of our preliminary assays using EBV receptor-positive Raji cells. The second preparation was virus from the P3HR-1 line. To obtain this preparation, P3HR-1 cells were incubated at 37 C for 3 days and then transferred to 34 C for 10 days. The culture fluid was then made cell free by low-speed centrifugation and membrane (Millipore Corp.) filtration. The virus was then pelleted at 75,000 × g per h (Spinco SW27 rotor) and resuspended at 2% of initial volume in RPMI with 10% FBS. This virus preparation was titrated and stored as described (12).

**Assay for surface-bound immunoglobulin.** Polyvalent, goat anti-human (immunoglobulins G, M, and A) immunoglobulin conjugate from a commercial source (Hyland Laboratory) was used diluted to 1:20 in direct immunofluorescent tests with fresh, live cells as described (8). At this dilution the conjugate stained very clearly 100% of BJA-B cells but failed to stain Molt 4.

**Lymphocyte purification and culture.** Unless otherwise specified below, CBL and adult peripheral blood lymphocytes (APL) were purified on a Ficoll-isopaque gradient (1) within 6 h after blood collection. Before using for any test or culture, lymphocytes were washed twice in phosphate-buffered saline (PBS, pH 7.2) or RPMI, respectively. For further lymphocyte purification the iron carbonyl technique was used as described (8).

For lymphocyte cultures, $10^6$ cells per ml of growth medium (RPMI + N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid buffer at 8 mM final concentration + 20% FBS + antibiotics as above) were cultured as described (12).

**Preparation of T and B lymphocyte populations by an anti-immunoglobulin column.** The presence of surface-bound immunoglobulin on lymphocytes detectable by membrane immunofluorescence is a B cell marker, whereas the spontaneous rosette formation with SRBC is a T cell marker (3, 9, 25). Therefore, in the present experiments we considered only these two exclusive markers for the definition of B and T lymphocytes.

Enriched T cell populations were prepared by the procedure of Wigzell et al. (26) as reported (8). T cell preparations containing more than 2% contaminant B cells (with surface immunoglobulin) were discarded.

To prepare B cell populations, purified peripheral

TABLE 1. *EBV adsorption on human lymphocytes as determined by membrane immunofluorescence (MI)*

| Lymphocytes[a] | Expt no. | Percentage of MI-positive cells[b] | | | | Control (Raji) |
| | | Unfractionated[c] | | Fractionated[j] | | |
| | | Untreated[d] | Treated | T lymphocyte population | B lymphocyte population | |
|---|---|---|---|---|---|---|
| CBL | I | 5.5 | 5 | 0 | ND[e] | 96 |
| | II | ND | 4.5 | 0 | ND | 96 |
| | III | 5 | ND | 0 | ND | 93.5 |
| | IV | ND | 5 | 0.5 (c) | ND | 91 |
| | V | 4 | 3 | 0 | ND | 91 |
| | VI | 6.5 | 6 | 0 | ND | 95.5 |
| APL(S−) | I (a) | ND | 5 | 0.75 (d) | ND | 92 |
| | II (a) | ND | 6 | 0 | 7.5 | 94.5 |
| APL(S+) | I | ND | 5.5 | 0 | ND | 93.5 |
| | II (b) | 6.5 | ND | 0 | ND | 97 |
| | III (b) | 6 | 4.5 | 0 | ND | 91 |

[a] CBL, Cord blood lymphocytes; APL(S−), peripheral blood lymphocytes from a healthy EBV-seronegative adult; APL(S+), peripheral blood lymphocytes from a healthy EBV-seropositive adult. Preparations (a) and (b) are from the same EBV-seronegative and EBV-seropositive donors, respectively, who were bled on two different occasions.

[b] For percentage of MI-positive cells, at least four different microscopic fields were first screened and then 200 cells were counted per sample except in (c) and (d), where 400 cells were scored. HRIK EBV (see text) was used in all these experiments.

[c] Fractionated and unfractionated here refers to T and/or B lymphocyte separation by using an anti-immunoglobulin column method. 0.5 to 2% of contaminating B cells were detected in T cell preparations reported in this table.

[d] "Treated" and "untreated" refers to whether the phagocytic cells were removed by the use of iron carbonyl powder (see text).

[e] ND, Not done.

blood lymphocytes were adsorbed into a Sephadex G-200 matrix covalently coupled with purified rabbit anti-human immunoglobulin antibodies. The serologically adsorbed B cells were recuperated after digestion of the mixture with dextranase as described elsewhere (23).

**Preparation of T lymphocytes from SRBC rosettes.** Equal parts of 1% SRBC and lymphocyte suspensions were mixed in FBS and treated for SRBC rosetting as described earlier (8). After the final incubation (4 C) for rosette formation for at least 2 h (the maximum period was an overnight incubation), the cells were gently lifted, overlayered on a Ficoll-isopaque gradient, and centrifuged at $400 \times g$ for 30 min. The pellet was then transferred to a tube, and a microsample was gently added in a small tube with trypan blue dye and examined with the microscope to assess rosette formation and cell viability. Only samples with greater than 99% viable rosette-forming cells (RFC) and lacking viable non-rosette-forming cells were further processed for T lymphocyte preparation as follows. SRBC from the pellet (containing also RFC) were lysed with a hypotonic solution (1 part of RPMI with 20% FBS in 4 parts of double-distilled water). The cells were then washed twice in PBS and, whenever possible (due to a very limited final number of cells), a small sample ($1 \times 10^5$ to $2 \times 10^5$ cells) was taken for surface immunoglobulin staining test. It may be important to mention here that, in another study where

six different lymphocyte preparations were obtained by this method, all were found to be free of contaminating surface immunoglobulin-positive cells and when these samples were examined by scanning electron microscopy it was also observed that all the cells had a rather smooth surface structure (Menezes, Graham, and Pederson, manuscript in preparation) compatible with the one described for human T cells (18). In the best circumstances, the total number of purified T cells obtained by this above method was about 3 to 5% of the initial lymphocyte population in the sample.

**Preparation of an RFC-DL population.** After separation of RFC as above, the cells at the interface layer were collected and a sample was examined for the presence of rosettes. Only those preparations with less than 10% contaminating RFC were treated by the hypotonic solution (see above), washed, and used as RFC-depleted lymphocytes (RFC-DL). It should be noted here that it was extremely difficult to obtain preparations with $0.5 \times 10^6$ cells of an RFC-DL population containing less than 10% contaminating RFC, particularly from cord blood samples that originally averaged not more than 10 to 15 ml.

**Assays for virus adsorption on lymphocytes.** Since a study on EBV receptors using P3HR-1 strain had already been reported (8), we decided to investigate virus adsorption on lymphocytes with this same strain of EBV. Undiluted and diluted

virus preparations were first assayed on indicator (control) Raji cells, and the virus dilution giving the highest percentage of fluorescent Raji cells with no appreciable background was then used throughout. Lymphoid cells were first sedimented by low-speed centrifugation and, after discarding the supernatant, EBV was added to the pellet (at a virus/cell ratio of 0.1 ml of EBV suspension/$10^5$ cells). The cells were then gently dispersed and the mixture was incubated at 4 C for 1 h. Uninfected control cells were treated in parallel with medium only. They were then washed twice with PBS and stained for direct membrane immunofluorescence as described before (10) by using reference fluorescein isothiocyanate-labeled (FITC) Abwao immunoglobulin diluted to 1:4 (8).

**Lymphocyte infection by EBV and EBNA kinetics.** CBL preparations from only very fresh blood samples (within less than 4 h after collection) were used in these experiments. After lymphocyte separation by a Ficoll-isopaque gradient as above, CBL preparations were briefly treated with a hypotonic solution (RPMI growth medium diluted 1:4 in sterile double-distilled water), washed once in RPMI with 25% FBS, and then incubated in growth medium at 37 C for 14 to 20 h. Only those CBL preparations that appeared to be in excellent condition when examined with an inverted microscope and which contained less than 5% nonviable cells (as determined by the trypan blue dye exclusion test) were used for EBNA kinetics as follows. Lymphocytes were pelleted by low-speed centrifugation and 1 ml of virus suspension (1 part of B95-8 virus + 1 part of RPMI medium with 30% FBS and antibiotics) was added per $10^6$ lymphocytes in the pellet. The tube containing this virus-cell mixture was then transferred to a 37 C humidified incubator with 5% $CO_2$. The mixture was gently shaken at every 15-min interval during the first hour of incubation. Uninfected controls were treated in parallel with growth medium only.

After an appropriate period of incubation, the cells were harvested to prepare smears for EBNA staining by the anti-complement immunofluorescence method (22), with the modifications given below. Cells were first washed twice in PBS and suspended for about 3 to 5 min in a 1:1 mixture of PBS-distilled water. Smears were prepared with approximately $10^5$ cells per cover slip, dried in air at room temperature, and fixed in a 1:1 mixture of cold acetone-methanol at −20 C for 3 min. The smears were then kept at −20 C until used.

Anti-complement immunofluorescence tests were carried out by using the following reagents: (i) reference JM serum with anti-EBNA + early antigen − virus capsid antigen + antibody composition derived from a healthy donor (22) (fresh JM serum was dispersed in 0.2- to 0.5-ml amounts in small plastic tubes and kept frozen at −90 C until used); (ii) FITC-conjugated goat anti-human $B_1C/B_1A$ immunoglobulin (Hyland Laboratories). For EBNA reactions, smears were incubated at room temperature for 45 min with non-inactivated JM serum at a dilution of 1:5 (as a common source of anti-EBNA antibody and complement) and then washed twice with PBS and incubated for 45 min at room tem-

perature with anti-human $B_1C/B_1A$ FITC conjugate. The smears were subsequently washed three times, counterstained with Evans blue, washed three times, and mounted on a slide in a 1:1 mixture of glycerol-PBS. Controls included EBV genome-positive Raji cells and genome-negative Molt 4 or BJA-B cells, heat-inactivated (56 C for 30 min) JM serum, and a fresh O serum, without anti-EBV antibody (derived from a healthy EBV-seronegative donor).

Smears of APL, when used for the EBNA kinetic study, were also treated as above.

## RESULTS

**EBV adsorption on lymphocytes.** In preliminary experiments it was found that EBV attachment to the cells can take place at 4 C as well as at room temperature of 37 C. However, in the case of CBL and APL, we found about 1 to 3% more nonviable cells in preparations that were incubated at 37 C as compared with those incubated at 22 or 4 C. Thereafter, all the assays for EBV adsorption on lymphocytes were regularly carried at 4 C (see Materials and Methods).

Under the experimental conditions described above, virus adsorption assays using unfractionated human blood lymphocytes of several donors and different origins − CBL and APL − gave similar results. Thus, it was found that about 5% of human blood lymphocytes adsorbed P3HR-1 EBV as judged by direct membrane immunofluorescence (Tables 1 and 2). On the other hand, T lymphocyte preparations, particularly those without detectable contaminating B cells, did not adsorb EBV on their surface (Tables 1 and 2). As expected, T lymphocyte preparations obtained by using two different types of separation techniques − anti-immunoglobulin column and gradient density separation of RFC − gave concordant results. When RFC-DL populations were used for virus adsorption tests, EBV attachment to 19 to 26.5% of them was observed (Table 2). These RFC-DL preparations still contained 5 to 7% of detectable RFC.

Fetal thymocytes did not appear to have receptors for EBV, whereas about 17% of liver lymphoid cells, 9.5 to 12% of spleen lymphoid cells, and 88% of control cells adsorbed the virus (Table 3).

**Lymphocyte transformation.** EBV-induced transformation was characterized by the following sequence of events: blast transformation of infected lymphocytes, followed by the proliferation of these blast cells and the establishment of a permanent cell line(s) with 90 to 100% EBNA-positive cells. Surface-bound immunoglobulin and rosette formation with SRBC (see Materials and Methods) were the only properties used during the present study for the

TABLE 2. *EBV adsorption on RFC and RFC-DL lymphocyte populations as compared with unfractionated lymphocytes from the same donors and Raji cells[a]*

| Cell origin[b] | Expt no. | Percentage of MI-positive cells | | | |
|---|---|---|---|---|---|
| | | Unfractionated cells | RFC | RFC-DL[c] | Raji |
| CBL | I | 4.5 | 0 | 19 (7) | 94 |
| APL(S−) | II | 6.5 | 0 | 26.5 (5) | 90 |
| APL(S+) | III | 6 | 0 | 23 (5) | 89.5 |

[a] P3HR-EBV (see text) was used for these experiments. 200 cells were scored to calculate percentage of membrane immunofluorescence (MI)-positive cells.

[b] Abbreviations are as in Table 1.

[c] Numbers in parentheses represent the percentage of contaminating SRBC RFC as detected after an overnight incubation at 4 C as for T cell rosette formation (see text).

TABLE 3. *EBV absorption on lymphoid cells from fetal thymus, spleen, and liver as determined by membrane immunofluorescence (MI)[a]*

| Percentage of MI-positive cells[b] | | | | | |
|---|---|---|---|---|---|
| Thymocytes | | Spleen cells | | Liver cells[c] (Donor I) | Control (Raji) |
| Donor I | Donor II | Donor I | Donor II | | |
| 0 | 0 | 9.5 | 12 | 17 | 88 |

[a] Lymphoid cells were prepared by scratching fragments of the respective organs against a nylon net (tightly attached over the mouth of a small beaker) and collected in RPMI. The cell suspension was then overlayered on a Ficoll-isopaque gradient and centrifuged as for lymphocyte separation (see text).

[b] Two hundred cells were scored for each sample. P3HR-EBV was used in all tests.

[c] Due to a technical mishap, liver cells of only one of the two donors were available for this test.

characterization of EBV-transformed LCL in terms of surface markers.

EBV induced lymphocyte transformation in almost all the cultures of T cell preparations that had detectable contaminating B cells, whereas no transformation was observed in T cell cultures without detectable B lymphocytes (Table 4). Furthermore, in our hands thymocyte cultures were not transformed by EBV.

None of the lymphoblastoid lines obtained after EBV transformation formed spontaneous rosettes with SRBC and, with the exception of one (ES-B-1), all of them carried surface-bound immunoglobulins detectable by direct immunofluorescence. The ES-B-1 cell line (Table 4) was derived from a T cell-enriched population separated from peripheral blood lymphocytes of a healthy, EBV-seropositive adult by the use of an anti-immunoglobulin column procedure (see Materials and Methods).

Following a recent report by Pope et al. (20) suggesting that lymphocyte transformation by EBV may require cell cooperation, we also infected with EBV adherent cell populations available at different times in our laboratory. After removing the nonadherent cells by three gentle washings with the medium, we infected adherent cell cultures (in Falcon flasks, no. 3012) from 10 different donors. The culture ages (before EBV infection) of these populations ranged from 28 to 60 days. EBV-induced transformation was observed in all these different cultures within 14 to 28 days of infection. Four to ten days after EBV infection, blastlike cells and their clumps formed on many of the adherent cells and a cell suspension grew gradually in each case, giving rise to an LCL. Control cells (uninfected) degenerated and died within 4 to 8 weeks. Cells of all these LCL contained EBNA, had surface-bound immunoglobulin, and did not form SRBC rosettes.

**Kinetics of EBNA appearance.** Because of the limited number of CBL obtained from each donor and the variable quality of these cells from different donors, we preferred to investigate the kinetics of EBNA appearance by using cell samples from several donors. We have thus found that EBNA can be detected in CBL at 18 h after EBV infection. Figure 1 gives the number of donors where EBNA was found at different periods. We disregarded the samples (donors) where the quality of smears was not considered technically adequate to assess with certainty the presence of EBNA and/or the percentage of positive cells. In two out of five samples the presence of EBNA was suggestive at 16 to 17 h after infection. The intensity of EBNA immunofluorescence increased progressively from 18 to 36 h. There was very little increase in percentage of positive cells between 18 and 24 h, but their number doubled between 26 and 30 h.

During the establishment of an LCL from EBV-infected CBL or APL cultures, we found that the increase in EBNA-positive cells reached its peak between 2 and 4 weeks, depending mainly on the donor cells. For a more precise determination of the increase of EBNA-positive cells during the establishment of such

TABLE 4. *Results of EBV infection of T lymphocyte preparations from CBL, from APL(S−) and APL(S+) healthy adults, and from fetal thymus*

| Lymphocytes (origin)[a] | Method of separation[b] | No. of donors | No. of cultures infected[c] | Contaminating B cells | No. of transformed cultures | Features of transformed cells | |
|---|---|---|---|---|---|---|---|
| | | | | | | SRBC rosettes | Surface immunoglobulin |
| CBL | I | 8 | 11 | 0.5–3% | 10 | − | + |
| | II | 5 | 5 (a) | 0 | 0 | | |
| APL(S−) | I | 2 | 2 | 0.5–2% | 2 | − | + |
| | II | 1 | 2 | 0 | 0 | | |
| APL(S+) | I | 2 | 2 | 1–2% | 2 | − | ±(=ES-B-1)[c] |
| | II | 3 | 4 (b) | 0 | 0 | | |
| Thymus | | 3 | 6 | ND [d] | 0 | | |

[a] Abbreviations are as in Table 2. Method I: by anti-immunoglobulin column; II: by separating rosette-forming cells on ficoll-isopaque gradient (for details see "Materials and Methods"). For thymocyte preparation, see note under Table 3.

[b] Wherever possible, two cultures (per donor) of lymphocyte preparation in tubes were infected with EBV. One noninfected culture of unfractionated lymphocyte population per donor was used as control for transformation. With the exception of cultures from two EBV-seropositive donors, no transformation was observed in these uninfected controls; EBV-induced transformation was, however, observed in the other control cultures of infected, unfractionated lymphocyte populations from all donors. T-lymphocyte samples from one donor in (a) and one donor in (b) were also tested for virus adsorption using P3HR-EBV, but the results were negative.

[c] Cells of ES-B-1 line express no surface-bound or intracellular immunoglobulins detectable by immunofluorescence and lack receptors for SRBC and Fc (immunoglobulin G), but about 62% of them have receptors for complement (Menezes et al., in preparation).
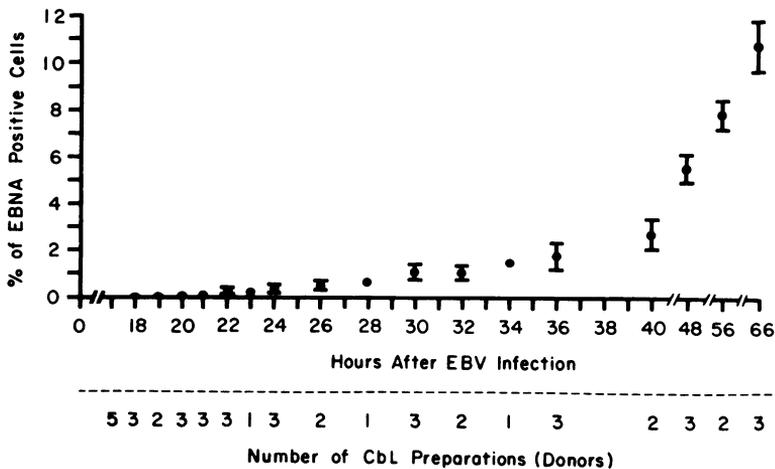
[d] ND, Not done.



FIG. 1. *Kinetics of expression of EBNA in CBL after infection by B95-8 EBV.*

an LCL, we infected a B cell population from an EBV-seronegative donor. There were about 85% of EBNA-positive cells in the culture 14 days after EBV infection, and their number reached over 90% at the end of the 3rd week (Fig. 2). Taking into consideration that in an EBV genome-positive LCL 85 to 100% of cells contain EBNA, it appears clear that the above B lymphocyte population (Fig. 2) became an LCL by the 3rd week after EBV infection.

Smears (two smears per culture) from lymphocyte cultures prepared during the 1st week (5 to 7 days) after EBV infection and stained

for the detection of early antigen and virus capsid antigen by immunofluorescence as previously described (12) were all found negative.

The appearance of EBNA and lymphocyte transformation were both prevented by treating virus preparations by serum with anti-EBV antibodies, heat, ether, and ultraviolet irradiation, as reported earlier (12).

## DISCUSSION

The present data show that thymocytes and T cell preparations lacking detectable contaminating B cells did not adsorb EBV on their

membrane. This supports the observation of Jondal and Klein (8) that T lymphocytes may lack EBV receptors on their surface. Our results also indicate that only a small fraction of human blood lymphocytes, and possibly less than 31% of B cells, carry receptors for P3HR-1 EBV. However, while this manuscript was in preparation, Greaves et al. (5) reported that virtually all human B lymphocytes have binding sites for EBV. Most probably these differences in results on B cells are due to the reagents used, mainly the virus preparations; in fact, different EBV strains were used by each group. It is possible that significant differences in binding to lymphocytes exist between the different EBV strains used, although no detectable differences were found between them at the serological level (2). They are, however, known to differ considerably in several of their biological properties (12). In any event, it is important to note here that it was previously suggested that P3HR-1 EBV binds poorly to human lymphocytes (8). The possibility that their technique is more sensitive and thus can detect the receptor-bearing cells more efficiently cannot be overlooked. In our hands the positive lymphocytes often showed weak fluorescence, generally with one to four very small fluorescent dots. Although the incubation periods for the staining were twice as long in our procedure, in the latter the cells were also washed one more time at each of the two main phases, i.e., first after incubation with the virus and second after incubation with the anti-EBV conjugate. On the other hand, under these conditions over 90% of the receptor-positive indicator (control) Raji cells showed membrane immunofluorescence, generally with two to seven dots and often brighter than those found on lymphocytes. These results indicate that further research is needed to investigate the differences regarding EBV adsorption on lymphocytes between or among different virus strains, particularly between lymphocyte-transforming and nontransforming strains of EBV.

The above findings that EBV-induced transformation was not observed in B cell-free lymphocyte preparations and in thymocyte cultures indicate that B cells (or their precursors), besides having the receptors for, can also be transformed by EBV. This agrees with the most recent work of others (17, 24, 27). However, the other possibility, although improbable, is that if T cells (or their precursors) are transformed by EBV, the resulting lymphoblastoid cultures may exhibit characteristics of B cells in vitro. However, taken together, the results of all our infection experiments with T cell cul-
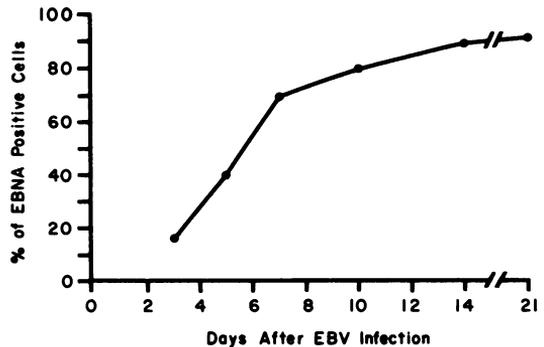


FIG. 2. *Kinetics of expression of EBNA during the establishment of a lymphoblastoid cell line. A B cell population, obtained by the use of a digestible anti-immunoglobulin column method from peripheral blood lymphocytes of a healthy EBV-seronegative adult donor, was infected with B95-8 EBV.*

tures suggest that if this is the case, it may indeed be a very rare event.

The case of ES-B-1 LCL is, however, most interesting. Since this line has no T cell property, lacks the main B cell marker (i.e., surface-bound immunoglobulin), and was found to express only complement receptors but not Fc receptors (Menezes et al., manuscript in preparation), it may well be derived from an atypical B lymphocyte or B cell precursor or from a so-called "null-cell." Since ES-B-1 was derived from a lymphocyte subpopulation of an EBV-seropositive individual after an in vitro EBV infection, it is presently impossible to demonstrate whether the line really was derived from a cell(s) that already carried the virus genome in vivo or whether the LCL was the result of an in vitro EBV infection.

We have also found that LCL can readily be obtained by infecting pure adherent cells with B95-8 EBV. The fact that these LCL have the characteristics of B cells implies that the adherent cells that were transformed were B cells. Present observations, although limited, suggest that cell cooperation (20) may not be necessary for B lymphocyte transformation by B95-8 EBV. This also agrees with the recent results of Yata et al. (27).

Our data on EBNA appearance in EBV-infected CBL show that it is the only EBV-induced intracellular antigen to be expressed before or during lymphocyte transformation by EBV strain B95-8. Moreover, whereas previous evidence indicated that EBNA would appear within 1 to 3 days after EBV infection (11, 16, 27), the present results clearly show that under appropriate conditions EBNA can be detected in lymphocytes as early as 18 h after infection. This early appearance of EBNA and the fact

that this antigen is also found in 85 to 100% of cells in an EBV-transformed LCL indicate that EBNA may be equivalent to the T antigen induced by oncogenic deoxyribonucleic acid viruses (e.g., papovaviruses). The main difference between these two nuclear antigens in the respective cell systems appears to be that the EBNA-positive lymphocytes grow into EBV-transformed LCL, whereas in the papovaviruses only a very low proportion of T antigen-positive cells may give rise to a transformed culture(s).

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