

## EPSTEIN-BARR VIRUS SUSCEPTIBILITY OF NORMAL HUMAN B LYMPHOCYTE POPULATIONS\*

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The Epstein-Barr virus (EBV)<sup>1</sup> is a human lymphotropic herpes virus associated with Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (1, 2). EBV is also known to cause infectious mononucleosis (3). It converts normal human B lymphocytes to immortalized lymphoblastoid lines. In vitro primary infection is followed by the expression of the EBV-determined nuclear antigen (EBNA; reference 4), cellular DNA synthesis with blast transformation and induction of Ig secretion (5).

Titration experiments have shown that only ~20% of in vitro EBV-infected peripheral blood B lymphocytes express EBNA (6). Several publications have reported attempts to characterize the EBV-susceptible subpopulation of B lymphocytes. The virus receptor is closely associated with the B lymphocyte C3d-receptor (7, 8). Using density gradient fractionation, Robinsson et al. (9) showed that EBV induced DNA synthesis only in B lymphocytes of high to intermediate density, but these authors did not study the EBNA expression. Einhorn et al. (10) reported that activation of B cells with protein A inhibited the transformation of B lymphocytes. According to these reports, the virus-susceptible subpopulation probably corresponds to the small resting C3d-receptor positive B cells.

EBV-transformed lymphoblastoid cell lines of nonneoplastic origin (LCL) have normal diploid karyotype (11). Such lines are polyclonal and their cells variable in size, morphology, and Ig expression (12). In contrast, cell lines derived from BL tissues are monoclonal and never have a normal diploid karyotype. In virtually all histologically proven cases, they were found to carry one of three specific chromosomal translocations, the 8;14 or the variants 8;2 or 8;22 (13). The cells are highly uniform, although different cell lines can differ (12). Both tumor and normally derived cell lines consists of large blastoid cells, but the morphology and differentiation marker expression shows a number of differences. The membrane glycoprotein pattern expressed by LCL is similar to mitogen-activated mature normal B lymphoblasts, whereas tumor lines exhibit a pattern comparable to small resting B lymphocytes (14). The LCL cells often express mIgD and secrete Ig at a high rate, whereas the BL lines with few exceptions are m-IgD

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<sup>1</sup>Abbreviations used in this paper: BL, Burkitt's lymphoma; BSS, buffered salt solution; EBNA, Epstein-Barr virus-determined nuclear antigen; EBV, Epstein-Barr virus; FCS, fetal calf serum; LCL, lymphoblastoid cell lines; NCS, newborn calf serum; PWM, pokeweed mitogen; SE, sheep erythrocytes; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

negative and have a very low Ig secretion (15). It is therefore believed that BL cells represent an immature stage of B cell development compared with the LCL.

The susceptibility of the different B cell subpopulations to EBV is still an open question. We have used density gradient fractionation to further investigate the problem. The expression of the EBNA was assessed as a marker for viral transformation. Virus binding and penetration were assayed by using fluorescein- and radio-labeled virus preparations. We have also examined the density gradient distribution profiles of two EBV-negative BL lines and their EBV-converted sublines in the comparison with the density of normal B lymphocytes during transformation *in vitro*.

### Materials and Methods

**Cell Separations.** Mononuclear cells were obtained from buffy coat by Ficoll-Isopaque centrifugation (16), washed, and incubated with carbonyl iron for 1 h at 37°C. Iron-ingesting cells were removed with a magnet. To separate T and B lymphocytes, the cells were mixed with 5% sheep erythrocytes (SE) in 20% newborn calf serum (NCS) and 3% dextran T70 (Pharmacia, Uppsala, Sweden) to form rosettes. After 45 min on ice, the rosetting and nonrosetting cells were separated on Ficoll-Isopaque gradient centrifugation (17). The nonrosetting B lymphocyte fraction was incubated for 1–2 h in flatly laying tissue culture bottles to remove adherent cells.

Cells from tonsils were prepared by mincing the tissue through a fine metal mesh. B cells were purified from the cell suspension as described for the blood mononuclear cells.

**Density-gradient Separations.** An isotonic stock of Percoll (Pharmacia) was prepared by mixing nine parts of Percoll with one part 10-fold concentrated buffered salt solution (BSS) (1.2 mM CaCl<sub>2</sub>, 138 mM NaCl, 5.4 mM KCl, 8.1 mM MgSO<sub>4</sub>, 0.5 mM KHPO<sub>4</sub>, 0.3 mM NaPO<sub>4</sub>, pH 6.9) and adjusting the osmolarity with 9% NaCl or water. This stock solution was mixed with BSS to 65.0%, 60.0%, 55.0%, 52.5, 50.0%, 47.5%, 45.0%, 42.5, and 40.0% Percoll. 1.5-ml portions of the nine solutions were layered over each other with decreasing density in a 15-ml tube to form noncontinuous nine-step gradients. Three-step gradients were prepared in a similar way using 3-ml volumes of 65.0%, 52.5%, and 42.5% Percoll solutions. The cells were suspended in 1 ml fetal calf serum (FCS) and layered on the top of the gradient. The gradient was centrifuged for 30 min at 800 *g* in a cooled swing-out rotor and the cell bands were collected with a pasteur pipette.

**Activation of B Cells.** Purified B cells were cultured in anti-IgM-coated plastic petri dishes for 80 h in medium with 10% FCS and 30% supernatant of pokeweed mitogen (PWM)-stimulated T cell cultures (18).

**Cell Lines.** The Ramos and BJAB lines have been established from EBV-negative lymphomas (19, 20). Two *in vitro* EBV-converted Ramos sublines, EHRA-Ramos (19) and Ramos-HR1K (21), and two converted BJAB lines, BJAB HR1K (21) and BJAB B95-8 (21), were also tested. The cell lines were resuspended every 3 or 4 d in tissue medium to a density of 0.2–0.3 million cells/ml. RPMI 1640 supplemented with 10% FCS and 100 U penicillin and 100 µg streptomycin/ml were used for all cultures.

**Virus.** Virus was prepared by concentrating 4 l of supernatant of the virus-producing B95-8 cells 400 times by centrifugation as described (22). The virus was stored frozen at minus 90°C and diluted 1:20 in medium with 10% FCS before use. Further concentration of the virus did not increase the percentage EBNA-expressing cells in infected normal B lymphocytes or Ramos cells. [<sup>3</sup>H]Thymidine-labeled virus was prepared after incubation of B95-8 cells with 10 µg/ml of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA; Sigma Chemical Co., St. Louis, MO) for 24 h to induce virus production (23). The cells were washed and incubated for 7 d at a starting density of 1 million/ml with 5 µCi tritiated thymidine per ml. The supernatants were collected and the virus was purified and concentrated as described above. Fluorescein labeling of the virus was performed as described by Johnson et al. (24). Concentrated virus of the B95-8 (25) and P3H (26) strains were supplied by Life Sciences, St. Petersburg, FL (grant provided by Shova

University Research Institute for Biomedicine in Florida). The virus was incubated with 20  $\mu\text{g}$  fluorescein per mg virus protein at a protein concentration of  $\sim 20$  mg/ml as determined by the Lowry method (27). The conjugation was performed at room temperature for 2.5 h. To separate the labeled virus from nonbound fluorescein, a Sephadex C 25-M column (Pharmacia) was used. The conjugates were stored at  $-90^\circ\text{C}$ .

*Virus Infection.* The cells were pelleted at 350  $g$  for 5 min and suspended in the virus solution at a density of  $1 \times 10^6$  cells/ml. Following incubation for 1 h at  $37^\circ\text{C}$ , the cells were washed and then resuspended in fresh medium at a density of  $0.5 \times 10^6$  cells/ml.

The virus binding assay and the assessment of [ $^3\text{H}$ ]thymidine-labeled viral DNA penetration was performed as described elsewhere (22). Briefly, 200  $\mu\text{l}$  of labeled virus was added to  $1 \times 10^6$  cells and incubated for 2 h at  $37^\circ\text{C}$  in a total volume of 1 ml BSS + 10% FCS. The samples were split into two equal parts, for binding and penetration assay. The cells were washed three times and the bound radioactivity was determined. To measure viral penetration of the plasma membrane, the cells were washed in trypsin buffer (BSS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) and resuspended in trypsin buffer with 0.25% trypsin. After 10 min of incubation at  $37^\circ\text{C}$ , the cells were washed three times in BSS + 10% FCS, and the cell-bound radioactivity was determined. The number of virus receptor-expressing cells was estimated by using fluorescein-labeled virus as described by Wells et al. (28).  $1 \times 10^6$  cells were incubated with 100  $\mu\text{l}$  of fluorescein-labeled virus diluted in BSS. After 30 min of gentle shaking at room temperature, the cells were washed three times in BSS and resuspended in BSS-glycerol 1:1. The samples were examined in a Leitz Orthoplan fluorescence microscope at 1,000 times magnification. EBNA staining was performed as described (29). At least 1,000 cells of each sample were counted in a fluorescence microscope at a magnification of 500. Control smears of all samples were stained with anti-EBNA-negative serum and smears from the EBNA-positive cell line Raji were stained and read in parallel as a positive control.

*Membrane Marker Staining.* Membrane Ig was detected by incubating  $0.5 \times 10^6$  cells in 50  $\mu\text{l}$  of fluorescein-conjugated rabbit anti-human IgM + IgG + IgA 1:10 (DAKO Immunoglobulins, Copenhagen, Denmark) on ice for 1 h. The cells were washed twice in BSS, resuspended in BSS-glycerol 1:1, and counted in a fluorescence microscope.

Staining with the pan-B-cell-reacting monoclonal antibody B1 (30) was performed by a two-step method.  $0.5 \times 10^6$  cells were suspended with the antibody at appropriate dilutions and incubated on ice for 30 min. The cells were washed, incubated with fluorescein-labeled rabbit anti-mouse Ig, and diluted 1:10 in BSS with 10% human serum on ice for 30 min. After a second wash the cells were resuspended in BSS-glycerol 1:1 for immunofluorescence examination. Controls were incubated with BSS followed by the second antibody. All membrane fluorescence stained samples were examined at 1,000 times magnification in the Leitz fluorescence microscope.

## Results

All B lymphocyte-enriched preparations were checked for SE rosetting and tested for membrane Ig and B1 expression. Blood-derived preparations contained 1–2% SE rosetting, 85–95% mIg-positive, and 90–95% B1-positive cells. Tonsil B cell preparations contained  $<0.5\%$  SE rosetting cells and  $>95\%$  membrane Ig-positive cells. Likewise,  $>95\%$  of these cells were B1-positive.

The density distribution of four purified peripheral blood B lymphocyte samples is shown in Fig. 1. B1-negative cells were enriched in the low density fractions (Table I). Blood-derived samples contained 10 and 35% B1-negative cells in the low density fraction 9. In the tonsil-derived samples the number of B1-negative cells were below 5% in all fractions. Only B cell preparations containing  $>75\%$  B1-positive cells in each fraction were used for experiments.

In order to test the EBV susceptibility, the cells from each fraction were

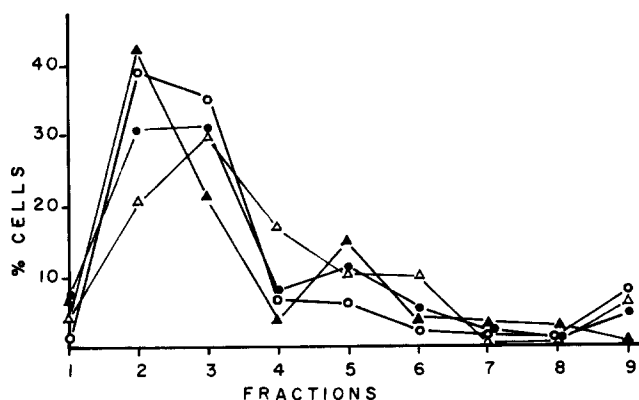


FIGURE 1. Distribution of human B lymphocytes in density gradients. Purified human blood or tonsil B lymphocytes were fractionated on nine-step density gradients and the cell number in each fraction was counted. The results are presented as the percentage of cells in each fraction of the total number recovered cells. Density is decreasing from 1 to 9. (A) Distribution of four samples of purified blood B lymphocytes. (B) Distribution of four samples of purified tonsil B lymphocytes.

TABLE I  
*Reactivity with the B1 Monoclonal Antibody in Fractionated Purified B Lymphocytes*

Fractions	Percentage fluorescent cells and standard deviation	
	Blood B lymphocytes ( <i>n</i> = 8)	Tonsil B lymphocytes ( <i>n</i> = 4)
9	82 ± 6	94 ± 4
8	86 ± 4	94 ± 4
7	84 ± 4	96 ± 2
6	92 ± 5	98 ± 2
5	94 ± 2	99 ± 0
4	96 ± 2	>99
3	>99	>99
2	>99	>99
1	>99	>99

Purified blood or tonsil B lymphocytes were fractionated on nine-step density gradients. The cells from each fraction were stained with the monoclonal antibody B1 and fluorescein-labeled rabbit anti-mouse Ig. The samples were then examined in fluorescence microscope and at least 500 cells from each sample were counted. Density decreases from 1 to 9.

collected, washed, and incubated with the B95-8 strain virus. The percentage of EBNA-expressing cells were counted after 48 h (Table II). EBNA-positive cells were found in the high density fractions 1–4 with some variations between the donors. The infectibility was confined to high density lymphocytes, as in earlier studies by Robinson et al. (10) using DNA synthesis as the measure of successful EBV infection.

In all subsequent experiments, three-step gradients were used. The first step corresponded to the first four fractions in the nine-step gradient and the second and third steps to fractions 5–7 and 8–9, respectively. In order to examine the

TABLE II  
*Frequency of EBNA-positive Cells in Different Percoll Fractions 48 h  
 after EBV Infection*

Fractions	Donor A	Donor B	Donor C	Donor D
9	0	0	0	0
8	0	0	0	0
7	0	0	0	0
6	0	0	0	0
5	0	0	0	0
4	2.0	0	0.2	0
3	2.4	8.0	0.5	0
2	6.1	2.0	0	1.4
1	6.1	1.1	3.0	3.4
Unfractionated	4.9	4.8	5.2	4.0

Purified human B lymphocytes from four donors were fractionated on nine-step Percoll gradients. Each fraction was incubated with (B95-8 substrain) EBV for 48 h before staining for EBNA. The EBV-negative cell line Ramos was used as a control of the infection and showed 13.5% EBNA-positive cells. At least  $2 \times 10^5$  cells from each sample were counted. Density decreases from 1 to 9.

possibility that the different infectibility of the various fractions might be determined at the receptor level, they were tested for virus using fluorescein or [ $^3\text{H}$ ]thymidine-labeled virus. Cell preparations pooled from five different blood donors or from tonsils were fractionated on three-step Percoll gradients. The fractions were exposed to labeled or nonlabeled virus to test virus binding and penetration after 2 h and EBNA expression after 60 h of incubation. Although certain differences were noticed in the frequency of EBV membrane fluorescence (EMF)-positive cells between different fractions, they were relatively modest and their significance is questionable (Table III). Similar results were seen in the tested tonsil samples.

Binding tests with tritiated methyl-thymidine-labeled virus showed that fractions 2 and 3 bound more virus than did fraction 1. Since fraction 1 contained between 36 and 23% EBNA-positive cells and fractions 2 and 3 contained little or no EBNA-positive cells, these results indicate that the presence of virus receptors on the membrane was not sufficient for transformation. In order to test whether the differences in EBNA expression could be explained by resistance to virus penetration of the plasma membrane in the low density fractions, we tested the uptake of labeled virus-DNA into the cells. The cells were incubated with [ $^3\text{H}$ ]thymidine-labeled virus for 2 h at  $37^\circ\text{C}$ , washed, trypsinated, and washed again to remove membrane-bound virus. The radioactivity in the cells was determined. Different incubation times with trypsin were tested. After 10 min of trypsination, no more radioactivity could be removed from the cells. The viability of the cells decreased after 15 min with trypsin. 10 min trypsination was therefore used in all experiments.

Our studies of density-fractionated lymphocytes showed that the virus penetration into fraction 2 cells was equal or slightly higher than into fraction 1, and the penetration of fraction 3 cells was clearly higher than into fraction 1 cells (Table III). Calculation of the percentage radioactivity of the total cpm bound

TABLE III  
*EBV Binding to and Penetration into B Cell Populations Fractionated on Three-step Density Gradients*

Sample	Fraction density	Per-centage EMF-positive cells	Binding of [ <sup>3</sup> H]thymidine-labeled virus		Virus penetration of the cells		Percent-age EBNA-positive cells 60 h after infection
			cpm	cpm	%	%	
Peripheral blood B lymphocytes	High	65					43
	Intermediate	69					<1
	Low	80					0
Tonsil B lymphocytes	High	66	5,337	4,750	90		36
	Intermediate	62	7,098	4,860	68		3
	Low	71	9,622	6,000	62		0
Tonsil B lymphocytes	High	69	4,899	4,063	83		23
	Intermediate	68	5,232	4,247	81		0
	Low	72	5,107	3,989	78		0
Controls	Raji	60	9,842	3,660	37		
	P3H	0	889	568	63		

Purified B lymphocytes from tonsils, or pooled from five blood donors, were fractionated on three-step density gradients into high, intermediate, and low density subpopulations. The virus binding was tested with fluorescein- and [<sup>3</sup>H]thymidine-labeled virus and the results presented as percentage EBV-membrane fluorescence (EMF) positive cells, or as cell-bound radioactivity (cpm). Penetration of viral DNA into the cells were estimated using [<sup>3</sup>H]thymidine-labeled virus and results are presented as cpm or percentage uptake of total bound radioactivity. The cells were incubated with 50,000 cpm radioactive virus. Cells infected in parallel were stained for EBNA 60 h after infection. At least 1,000 cells were counted for each sample. The EBV receptor-positive cell line Raji and the receptor negative cell line P3H were used as controls. Experiments using labeled P3H or B95-8 virus gave identical results.

to the cells, showed however that whereas 83–90% of the bound virus penetrated fraction 1 cells, 68–81% and 62–78% of the virus DNA was found inside the cells. The resistance to EBV-transformation in fractions 2 and 3 could therefore not be explained by different virus binding or virus penetration into these subpopulations.

The density of B cells decreases upon activation. It was therefore likely that the low density fractions represented activated B cells. Following this hypothesis, we have tested the infectibility of activated B cell populations. Tonsil-derived B lymphocytes were activated by culturing in petri dishes coated with rabbit anti-human IgM in the presence of 30% supernatant from PWM-stimulated 3-d-old T cell cultures. Controls were cultured in petri dishes without antibodies and T cell supernatants, or kept at 4°C. After 80 h, the cell suspensions were cleared of debris and dead cells by Ficoll-Isopaque centrifugation and subsequently fractionated on Percoll gradients. Both the antibody-stimulated and control cultured cells were distributed mainly in the low and intermediate density fractions. The majority of the stimulated cells were found in the low density fraction 3, while the control cultures were found mainly in fraction 2. The cells kept at 4°C showed a pattern similar to that of the original cell preparation.

Cells infected without prior cultivation became EBNA positive with a frequency of 10–26% (Table IV). In contrast, no EBNA-positive cells were found either in the stimulated or in the control cultures infected after 80 h in vitro culture. Infection after 80 h at 4°C in culture medium induced 4–11% EBNA-positive cells. The subsequent proliferation intensity and survival of the cultures was in line with the EBNA staining. EBNA-positive cells grew out only from the directly infected cells and from cells stored 80 h in a refrigerator.

EBV transformation of normal B lymphocytes leads to polyclonal cell activation, blast transformation, and in addition, prolonged persistence of EBV genomes to expression of EBNA and possibly other viral products as well. If one wishes to dissociate the effects of EBV in inducing blast transformation from the phenotypic effects due to persistence of the viral genome and presence of the nuclear antigen, EBV-negative B lymphoma lines and their in vitro EBV-converted sublines are useful, since they represent the same clone of immortal neoplastic blast cells that only differ from each other with regard to the presence of EBV-DNA and EBNA in the converted lines.

Previously, such comparisons have detected differences between the negative lines and their derived EBNA-positive sublines, with regard to sensitivity to saturation density (31), serum requirements, capping properties (32), lectin agglutinability (33), membrane IgD, and insulin receptor expression (34). No difference in density was seen, however, between EBNA-positive and -negative lines. In addition to these lines, we have also tested 16 EBV-carrying cell lines. 5 of these were lymphoblastoid lines (LCL) of normal origin, whereas 11 were BL lines. All established lines showed similar density distribution, restricted to the low density part of the gradient, i.e. fractions 6 to 9.

In view of the fact that the target cells of the transformation were only seen in the high to intermediate density fractions, we have examined the density of the EBNA-positive cells shortly after infection.

Purified B cells were fractionated on three-step gradients and the separated high intermediate and low density cells were infected with the B95-8 substrain of EBV. The cells were incubated at 37°C for 48 h and each fraction was once again fractionated on a three-step Percoll gradient. The fractionated cells were

TABLE IV  
*Frequency of EBNA-positive Cells in Directly Infected vs. Preincubated B Lymphocytes*

Preculture	Medium supplement during pre-culture	Percent EBNA-positive cells		
		Donor 1	Donor 2	Donor 3
None	—	10	23	26
80 h at 37°C	Anti- $\mu$ -serum + T cell factors	0	0	0
80 h at 37°C	None	0	0	0
80 h at 4°C	None	4	5	11

B lymphocytes were purified from tonsils and infected with EBV directly or after cultivation as indicated. The cells were harvested for EBNA staining 48 h after infection.

collected, washed, counted, and stained for EBNA. Samples from all fractions were also taken for further cultivation. As shown in Fig. 2, 73–83% of the high density population had shifted to intermediate density and 3–8% had shifted to low density (Fig. 2*B*). The same shift occurred without virus infection, however

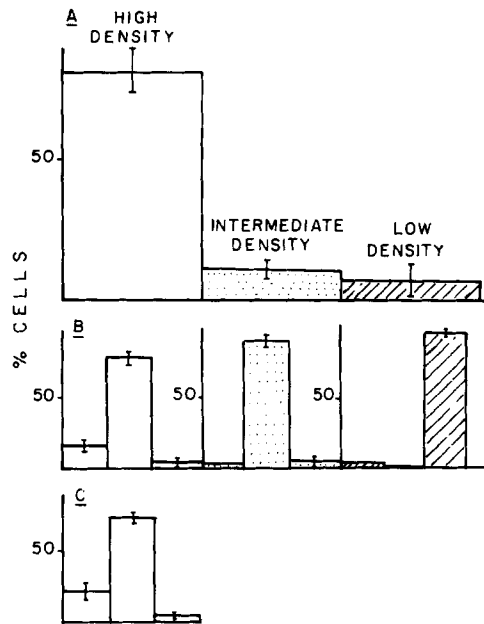


FIGURE 2. Distribution of human blood B lymphocytes in three-step density gradients before and after 40 h of incubation. (A) Distribution of four freshly prepared samples. (B) Distribution of the cells in each fraction after EBV B95-8 substrain infection and subsequent incubation for 48 h at 37°C. (C) Distribution of high density controls cultured 48 h at 37°C without EBV. The figure shows the mean percentage of cells in high density, intermediate, and low density fractions. Standard deviation is indicated.

TABLE V  
Percentage EBNA-positive Cells 48 h after the EBV Infection of B Lymphocytes of Different Densities

EBV-infected density fraction	Density fraction at harvest for EBNA staining	Percentage EBNA-positive cells			
		Donor A	Donor B	Donor C	Donor D
High	High	0	0	0	0
	Intermediate	16	14	13	18
	Low	0	1	3	0
Intermediate	Unfractionated	12	9	11	9
	Unfractionated	0	0	0	0
Low	Unfractionated	0	0	0	0

Purified B cells from blood were fractionated on three-step density gradients before and 48 h after EBV infection. Frequency of EBNA-producing cells in each fraction was determined. At least 1,000 cells from each sample were counted in immunofluorescence microscope. The EBV-negative lymphoma cell line Ramos was infected as a control. 24% of the Ramos cells were EBNA-positive 48 h after infection. Donors and fractions as in Fig. 2.



TABLE VI  
*EBNA Production in EBV-infected Purified High Density B  
 Lymphocytes Density Fractionated 9 h after Infection*

Density fractions	Donor 1	Donor 2	Donor 3
High	0	0	0
Intermediate	43	29	62
Unfractionated	14	16	19

High density B lymphocytes were purified from tonsils, infected with EBV, and incubated for 9 h before density fractionation on three-step gradients. The high and intermediate density cells were incubated for 40 h more, harvested, and stained for EBNA. No cells were found in the low density part of the gradient. At least 1,000 cells from each sample were counted in an immunofluorescence microscope. The EBV-negative cell line Ramos was control-infected and found to contain 18% EBNA-positive cells 50 h after infection.

(Fig. 2C). The intermediate and low density cells were more stable and showed only minor changes in their gradient distribution.

Table V shows the occurrence of EBNA-positive cells in the different subpopulations. Between 8 and 14% EBNA-positive cells were found in the initial high density population. The other populations remained negative. In the high density population, between 13 and 18% of the cells that had shifted to intermediate density and 0–3% of the cells that shifted to low density were found EBNA positive. In contrast, no EBNA-positive cells remained at high density. The subsequent proliferation history of the cultures was in line with the EBNA positivity. Proliferating cells were only seen in the samples where EBNA was detected, even when the observation period was extended to several weeks of incubation and nursing. These results showed that the EBNA expression was associated with a shift to lower density and that high density cells never expressed EBNA. In order to further investigate sequences of density shift and EBNA expression, we purified high density tonsil B lymphocytes, infected the cells with B95-8 strain of EBV, and incubated for 9 h at 37°C. The cells were again fractionated into high, intermediate, and low density fractions and each fraction was incubated further for 40 h at 37°C before harvesting and staining for EBNA.

The results show that only cells that had shifted from high to intermediate density 9 h after infection, later produced EBNA (Table VI). EBNA was reported to be produced at the earliest, 12 h after infection (5). Taken together, these results indicated that only cells that have changed from high to intermediate density within 9 h after infection, were later induced to EBNA expression. Thus, the cells had changed their density at least 3 h before the onset of EBNA production.

### Discussion

It is well known that EBV can only infect and transform a subpopulation of human B lymphocytes, but the characteristics of this subpopulation have not been established. New separation methods have allowed a fractionation of B cell populations that permits a detailed study of this problem. In the present study we fractionated purified human B lymphocytes according to their density. We

could then study the susceptibility of B cells of different density to EBV infection, as judged by EBNA synthesis and subsequent outgrowth of lines. We have also studied changes in cellular density that occurred in the course of EBV transformation. Three subpopulations of human blood and tonsil B lymphocytes were studied regarding their susceptibility to EBV: the high density fractions are enriched for small resting B cells (35, 36, Åman et al., unpublished observations). In the mouse system they have been described as Go cells, and both mouse and human high density B cells respond poorly to mitogens as judged by DNA synthesis (35, 36). Intermediate density cells were characterized in the mouse system as G1 cells. They give a strong DNA synthesis response after mitogen stimulation. The low density population is enriched for large activated cells. Some of them are already in the S-phase, as indicated by a spontaneous DNA synthesis (Åman et al., in preparation). The three density fractions also showed differences in expression of several B cell differentiation markers detected by monoclonal antibodies.

Our results showed that only cells in the high density fractions could be infected with EBV as indicated by EBNA synthesis. All fractions contained >60% EBV receptor-positive cells and the virus penetrated cells from all the fractions. The small variations in EBV receptor-expressing cells and virus penetration of the plasma membrane could not explain the striking differences in EBNA producers seen after infection. Since B1-negative cells were enriched in the low density part of the gradient (Table I), the question arises whether these cells could have inhibited the EBV infection. This is unlikely, however, since intermediate and low density fractions from tonsils contained no or few B1-negative cells and still were resistant to EBV transformation. Our results, therefore strongly suggest that only resting B lymphocytes can be infected by EBV. This is further supported by the results presented in Table IV, showing that T cells and macrophage-independent activation of B cells prevented their infectibility.

We also found that *in vitro* cultivation leads to a decrease in density and blocks the EBV susceptibility itself. This density shift does not appear to be an osmotic artefact due to hypotonicity of the medium or its relatively low protein content, since the process is inhibited at 4°C. Moreover, monoclonal B lymphocyte populations from chronic lymphocytic leukemia patients remained stable at high density during several days of *in vitro* cultivation (Åman et al., in preparation).

If it is accepted that the changes in density are due to activation, a number of alternatives can be considered as possible explanations for this phenomenon. Several factors may be considered as being potentially responsible for this spontaneous density shift. It could represent an *in vitro* continuation to a maturation process that may have started already *in vivo*. It might reflect nonspecific activation that may occur during preparation of the B cells, and it could be due to the heterologous serum component in the medium. The latter possibility has been excluded by results from experiments made in 10–30% autologous serum. In other experiments we have found that the majority of nonstimulated B cells during the first 24 h of cultivation express transiently the activation markers BBI and LB1 (37). This is in contrast with the anti-Ig + T cell supernatant stimulated cells that show a stable expression of the same activation markers.

The exclusive susceptibility of the heavy resting B cells to the EBV transformation contrasts with the low density of the EBV-transformed cell lines. Short-term experiments showed that the density shift precedes the EBNA production. This can be interpreted in two ways. The virus may infect only the very small population that is in the process of the activation/maturation-like event detected as a density shift, or the virus infects also other cells but the spontaneous activation-density decrease of the cells is necessary for initiation of the viral activity inside the cell. Further investigations of early events during activation and EBV transformation will show which alternative is right.

### Summary

Human blood and tonsil B lymphocytes were fractionated on density gradients and tested for virus binding and penetration into the cells. Epstein-Barr Virus (EBV) transformation was detected by immunofluorescence staining for EBV-determined nuclear antigen (EBNA). EBV bound to and penetrated all B cell populations, but only the high density populations were transformed. Activated B lymphocytes were found in the low density fractions and these cells were resistant to EBV infection.

Infected and noninfected B lymphocytes were density-analyzed during in vitro culture. A spontaneous, not virus-induced, density decrease was found to precede the production of EBNA. Cells remaining at high density never expressed EBNA.

The results suggest that EBV can transform only small resting B lymphocytes and that a virus-independent activation of the infected cells induces the EBNA production and transformation.

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