

EARLY EVENTS IN EPSTEIN-BARR VIRUS INFECTION PROVIDE A MODEL FOR B CELL ACTIVATION

BY DAVID A. THORLEY-LAWSON AND KAREN P. MANN

*From the Department of Pathology, Tufts University School of Medicine, 136 Harrison Avenue,
Boston, Massachusetts 02111*

B lymphocytes may be activated by a variety of agents, including antigens and mitogens. The minimum stage of activation so far demonstrated has been passage from the resting G_0 stage into an activated G_1 phase. This is operationally defined as blastogenesis, and is marked by increased RNA synthesis and expression of surface markers characteristic of proliferating cells in the absence of DNA synthesis (1-3). In the presence of appropriate soluble factors, these cells will proliferate, then terminally differentiate into antibody-secreting plasma cells.

Epstein-Barr virus (EBV)¹ infects resting B cells (4-9) and drives them to differentiate into Ig-secreting lymphoblasts (10, 11) that express cell surface markers characteristic of activated B cells (12, 13). These lymphoblasts proliferate indefinitely in culture (14, 15), a process that has been termed growth transformation or immortalization.

We have proposed (12) that growth transformation by EBV involves two processes. The first parallels the normal differentiation pathway for resting B cells whereby they become activated and proliferate. The second involves the fixing of the lymphoblasts at this stage, leading to indefinite proliferation. Thus, the mechanism of B cell activation is intricately involved with the process of EBV infection. In addition, EBV has several advantages over other systems, such as anti-IgM or mitogens, for studying the activation of resting B cells. There are clear-cut markers for the presence of the activating signal (EBV DNA, and virus-encoded antigens), and activation of a large fraction (up to 40%) (5, 16) of the B cells may be obtained with a fairly synchronous order of events. These include the induction of a viral nuclear antigen (EBNA) by 24 h, and blastogenesis, proliferation, and expression of p63 (an EBV-encoded membrane protein) (17 and Mann et al. submitted), between 48 and 72 h. Furthermore, no accessory cells are required, and EBV-infected cells are immortalized at a particular stage of differentiation, allowing access to bulk quantities of defined cells for biochemical analysis. We have previously used the EBV system to describe a B cell activation antigen, BLAST-1 (12). This marker was not found on resting B cells, but was detected on their surface when they underwent blastogenesis after

This work was supported by grants AI15310 and CA28737 from the U. S. Public Health Service (USPHS). D. Thorley-Lawson is the recipient of a Research Career Development Award AI 00549 from the USPHS. K. Mann was supported, in part, by training grant AI07077 from the USPHS.

¹ *Abbreviations used in this paper:* CLL, chronic lymphocytic leukemia; EBNA, EBV nuclear antigen; EBV, Epstein-Barr virus; EBVCS, EBV cell surface; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline.

activation by mitogens, antigen, or EBV. Using the EBV cell surface (EBVCS) monoclonal antibodies (mAb) (18), we have been able to describe a second activation antigen, BLAST-2 (EBVCS), which is also B cell-specific. However, this marker was different from BLAST-1, as it was detected within 24 h of the activation signal, well before the time of blastogenesis.

Our previous experiments were conducted on bulk cultures, and the level of expression and number of BLAST-2⁺ cells was low. Therefore, it was not possible to tell whether the BLAST-2 (EBVCS)⁺ cells present soon after the activation signal were the same cells that would subsequently undergo blastogenesis and proliferation.

In this report, we use the EBV system to study the BLAST-2 (EBVCS)⁺ and BLAST-2⁻ populations early after infection. We show that the BLAST-2⁺ cells are small lymphocytes, clearly distinct from proliferating lymphoblasts. Nevertheless, when these cells are placed back in culture, they proceed to undergo transformation into B lymphoblasts. Thus, we conclude that B cell activation proceeds through two distinct steps; the first occurs within 24 h, and is characterized by expression of the BLAST-2 (EBVCS) antigen and induction of RNA synthesis; the second occurs later, and is associated with blastogenesis, proliferation, and expression of BLAST-1. Furthermore, BLAST-2 (EBVCS) is the earliest B cell-specific cell surface marker so far identified for activated B cells.

Materials and Methods

Cells. Freshly transformed B lymphocytes were prepared as follows: 2–3 ml of packed buffy-coat cells were obtained by centrifugation of plasmapheresis by-product material (Red Cross, Boston MA) over Ficoll-hypaque. The cells were washed once in medium (RPMI 1640 plus 5% fetal calf serum), and B lymphocytes were isolated by passage over a column of rabbit anti-human Fab' coupled to Sephadex G-200 (19, 20). ~10⁹ cells were passed over each column of 12 ml packed bed volume. The column was washed thoroughly with medium plus 1 mM EDTA (column buffer), and the adhered B lymphocytes were then removed by stirring in the presence of column buffer supplemented with 1% human gamma globulin, Cohn Fraction II (Sigma Chemical Co., St. Louis, MO). Typically, 10–15% of the lymphocytes were recovered in the Ig⁺ population, yielding 2–3 × 10⁸ purified B cells. This population will be referred to as peripheral blood lymphocyte (PBL)-B cells, and was usually >90% B cells by the criteria of surface Ig and B1 (21) antigen expression, and <1% T cells using the T3 surface marker. These cells were infected at a density of 4 × 10⁶ cells/ml with EBV from the culture supernatant of the B95-8 lymphoblastoid cell line (TD₅₀ ~5 × 10⁵). TD₅₀ was estimated by limiting-dilution analysis on constant numbers of purified B cells. Our experiments show that such EBV-infected cells have a cloning efficiency of 1–2% on a feeder layer of irradiated human foreskin fibroblasts (350 Q). Thus our estimated TD₅₀ is ~50–100-fold lower than the actual number of infectious particles (i.e. ~10⁷ viruses/ml). Infection was either with undiluted supernatant, or with supernatant diluted 1:2 or 1:4 with medium containing 20% fetal calf serum. When undiluted virus was used, half of the volume was replaced with medium after 2 h at 37°C. The three dilutions of virus generally gave 12–18%, 7–12%, and 3–7% BLAST-2⁺ cells, respectively, at 36 h postinfection. Visual assessment was used to measure growth transformation; our criteria have been described in detail elsewhere (20). Briefly, a culture in a microtiter well was regarded as growth transformed if it contained large numbers of proliferating lymphoblasts, both as single cells and clumps. With the dilutions of virus supernatant used in these experiments (see above), growth transformation was always achieved within 10 d postinfection.

Cytofluorographic Analysis. Cells from infected cultures were washed twice with RPMI-1640 supplemented with 1% fetal calf serum (medium), and cells were resuspended in anti-BLAST-2 (EBVCS) hybridoma ascites fluid. The hybridoma used was EBVCS 2 (18),

kindly provided by Dr. B. Sugden, and shown previously in this laboratory to define the BLAST-2 (EBVCS) antigen (13) and the anti-BLAST-1 previously described by this laboratory (12). Fluorescence analysis revealed that these ascites had a titer of $1:10^4$ – 10^5 and $1:10^2$ – 10^3 , respectively, and were routinely used at 1:100 or 1:50 dilution, respectively, thus assuring saturation. Unreactive mAb of the same isotypes were used as negative controls. For analysis, 10^6 cells were resuspended in 100 μ l of antibody, and for sorting experiments, 2 – 3×10^7 cells were labelled in separate tubes, each with 200 μ l of antibody. The cells were then incubated on ice for 20 min, washed twice, and resuspended in 0.1 or 0.2 ml of fluorescein isothiocyanate (FITC)-labelled goat anti-mouse Ig (Cappell) diluted 1:20. After a further 20 min incubation at 4°C, the cells were washed twice, and resuspended either in 1 ml for analytical experiments (10^6 cells/ml), or 5 ml for sorting experiments (5×10^6 cells/ml).

For the analysis of cell cycle stage, the FITC-labelled cells were costained with propidium iodide. The labelled cells (10^6) were resuspended in 0.5 ml of phosphate-buffered saline (PBS), and an equal volume of 100% ethanol was added slowly while vortexing vigorously. Alcohol fixation was found previously (our unpublished observations) not to affect the fluorescence due to the labelled BLAST-2 (EBVCS) antigen. The cells were incubated for at least 1 h at 4°C, then washed once with PBS and resuspended in 250 μ l of 500 U/ml ribonuclease A (Sigma Chemical Corp.) in 1.12% sodium citrate buffer, pH 8.4. This mixture was incubated for 30 min at 37°C, and then an equal volume (250 μ l) of propidium iodide (50 μ g/ml) in 1.12% sodium citrate buffer was added and the mixture, incubated for an additional 30 min at room temperature. Specific RNA staining was performed exactly as described (22) by using the Hoechst 33342 and Pyronin Y dyes.

Analysis was performed on a fluorescence-activated cell sorter (FACS) analyzer, and sorting was done on a FACS III or FACS IV cell sorter. For cell size and cell cycle studies, the most fluorescent population was identified, and gates set accordingly. The size distribution or staining profile of this population was then assessed. For sorting experiments, only small lymphocytes were collected, to avoid contamination by lymphoblasts or monocytes. Usually, collecting the 20% most and least fluorescent cells gave the best yields and purity of the BLAST-2 (EBVCS)⁺ and BLAST-2⁻ populations. Generally, positive selection yielded a population >60% BLAST-2 (EBVCS)⁺, and negative selection gave a population <1% BLAST-2 (EBVCS)⁺. An example of such dual analysis is presented in Fig. 3. For sorting on the basis of RNA content, the high and low populations were separated (see Fig. 5), and the gates were set to collect whole populations. Cloning was performed using a Coulter autocloner, previously calibrated with fluorescent beads. Microtiter plates containing 1, 10, and 100 cells/well were established on a feeder layer of irradiated human foreskin fibroblasts (350 Q).

Hybridization. Cells were washed once with PBS and resuspended to 10^8 cells/ml. 10^6 cells were routinely spotted onto nitrocellulose (Bio-Rad Laboratories, Richmond, CA) as two 5- μ l aliquots, with drying in between. Thereafter, the procedure used was the colony hybridization method of Grunstein and Hogness (23), as modified by Brandsma and Miller (24) for lymphoid cells. The Bam H1 V fragment (kind gift of T. Dambaugh) was used as a probe.

Inhibitors. The concentrations of metabolic inhibitors chosen were the maximum necessary to obtain inhibition without affecting viability of an EBV-transformed lymphoblastoid cell line after 2 d of culture. The following inhibitors were used: cycloheximide (5 μ g/ml), actinomycin D (1 μ g/ml), arabinoside C (1 μ g/ml), and acyclovir (100 μ M). Inhibitors were added to culture, at the dosage described, 1 h before infection, and were maintained in the culture throughout the course of the experiment.

EBNA Stain. Staining for EBNA was carried out by the standard technique described previously by Reedman and Klein (25), using EBNA⁺ and EBNA⁻ sera characterized and kindly supplied by Dr. G. Klein (Karolinska Institute, Stockholm, Sweden).

Results

The BLAST-2 (EBVCS) Antigen is Expressed Before Transformation. We have shown previously (13) that the BLAST-2 (EBVCS) antigen is expressed on B

cells within 24 hours after EBV infection. This is well before the time of blastogenesis and proliferation, which occur 2–3 d postinfection (5, 26, 16). An example of such an experiment is shown in Fig. 1.

EBV-infected PBL-B were tested at various times postinfection for the expression of the BLAST-2 (EBVCS) antigen. The cells were stained with the anti-BLAST-2 (EBVCS) mAb, and analyzed by cytofluorography. Within 1 d postinfection, ~10% of the cells expressed the BLAST-2 (EBVCS) antigen. A typical example of the fluorescence profile obtained with cells at this time is shown in Fig. 2. The BLAST-2⁺ cells are clearly resolved as a distinct population. This population was absent from a negative control of the same cells stained with an unreactive mAb of the same Ig class as anti-BLAST-2 (EBVCS). Interestingly,

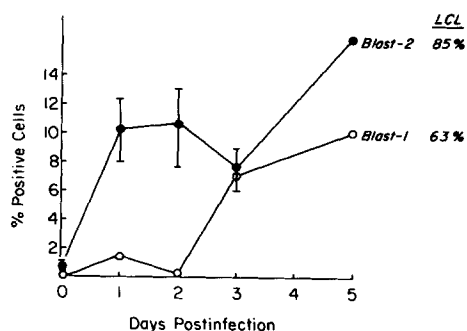


FIGURE 1. Expression of the BLAST-1 and BLAST-2 antigens on EBV-infected PBL-B cells. Freshly isolated PBL-B cells (4×10^6 cells/ml) were infected with B95-8 virus (tissue culture supernatant diluted 1:1 with medium) and placed in culture. Cells were recovered each day and stained with either anti-BLAST-1 or anti-BLAST-2 (EBVCS) mAb followed by FITC-conjugated rabbit anti-mouse Ig, and cytofluorographic analysis was performed. The percentage of positive cells was assessed by subtraction of background fluorescence obtained with a negative control mAb. The BLAST-2 (EBVCS) data represent means of three independent experiments. The BLAST-1 data is from a single experiment. LCL, established lymphoblastoid cell line.

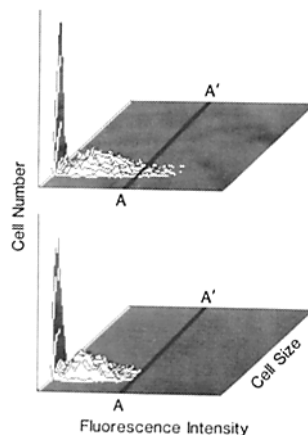


FIGURE 2. Fluorescence profile of PBL-B cells stained with anti-BLAST-2 (EBVCS) at 36 h postinfection. Infected cells were prepared and labelled as described in the legend to Fig. 1. Each histogram represents the analysis of 10,000 cells. An arbitrary cut off line (A-A') has been drawn to highlight the population of positive cells. (Top) stained with anti-BLAST-2 (EBVCS). (Bottom) stained with negative control mAb.

the three dimensional plot indicates that, at this time, the BLAST-2⁺ cells are of the same size as the bulk of the cells in the culture, which are small resting PBL-B cells (see below).

The time of appearance of B lymphoblasts was demonstrated by staining with the anti-BLAST-1 mAb (Fig. 1). As may be seen in Fig. 1, BLAST-1-reactive cells were not detected until 2 d after the appearance of BLAST-2 (EBVCS).

Expression of the BLAST-2 (EBVCS) Antigen Predicts Transformation. The experiments described previously and exemplified above lead to the hypothesis that expression of the BLAST-2 (EBVCS) antigen indicates that a cell is activated and will undergo blastogenesis and proliferation. To test this prediction, we separated the BLAST-2 (EBVCS)⁺ from BLAST-2⁻ populations, and placed them in culture. Infected B cells were labelled by indirect immunofluorescence using the BLAST-2 (EBVCS) mAb or an unreactive antibody of the same class. The 20% most and least fluorescent cells were isolated using a FACS III cell sorter under sterile conditions, and were placed back in culture alongside unfractionated cells. Upon reanalysis, there were 15% BLAST-2⁺ cells in the unfractionated population; 60% in the positively selected population, and <1% in the negatively selected population (Fig. 3). The cultures were assessed visually for growth transformation immediately after plating, and daily thereafter. Fig. 3 shows photographs of the three cultures taken immediately after separation (day 2), and 8 d later (day 10). The cultures were essentially indistinguishable when first plated; however, extensive overgrowth of the wells by proliferating lymphoblasts was seen only in the culture from the BLAST-2⁺ cells. Proliferation in the unseparated population was also seen, but at a much lower level, with complete outgrowth seen in all of the cultures only by the end of the experiment (day 10). In contrast, no outgrowth, and only an occasional lymphoblast was seen in any of the cultures from the BLAST-2⁻ cell population by the time the experiment was terminated.

Staining with the monocyte-specific antibody MO-2 (27) suggests (data not shown) that monocytes are the only major contaminating cell population (as high as 10%) of our purified B cells. It was, therefore, possible that our 20% least fluorescent population was enriched for monocytes, and thus would not have proliferated and overgrown the culture well. Three observations make this explanation unlikely: (a) a size gate characteristic of small lymphocytes was used for the sorting, and since monocytes are usually larger, they would have been excluded; (b) most monocytes would have adhered to the plastic dishes during the culture period; and (c) reanalysis revealed that the BLAST-2 (EBVCS)⁻ population of cells readily reacted with the B cell-specific antibody B1 (21), but no detectable reaction (<1%) was seen with the monocyte-specific antibody MO-2 (27), nor with the pan-T cell specific mAb T3.

A further, quantitative indication of the relationship between proliferation and BLAST-2 (EBVCS) expression was obtained by plating various numbers of BLAST-2 (EBVCS)⁺ and BLAST-2⁻ cells in microwells, and assessing the number of wells outgrown by day 10. As seen in Table I, no outgrowth of the negatively selected cells was seen at the highest cell number used, 5×10^5

CYTOFLUOROGRAPHIC ANALYSIS

APPEARANCE OF LYMPHOCYTES

IMMEDIATELY AFTER SORT

(2 d postinfection)

(7 d postinfection)

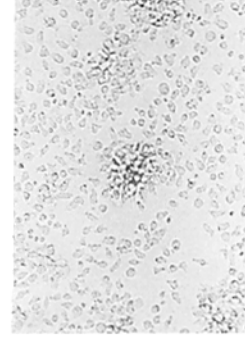
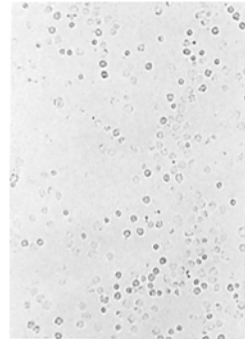
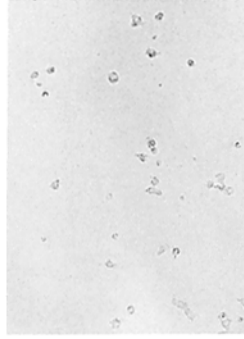
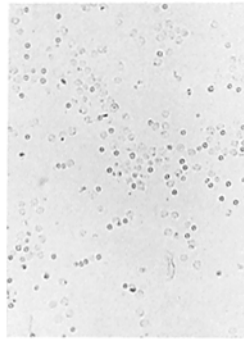
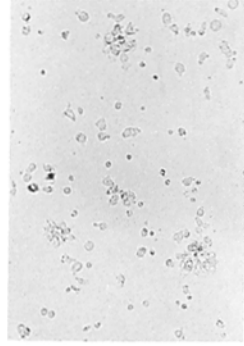
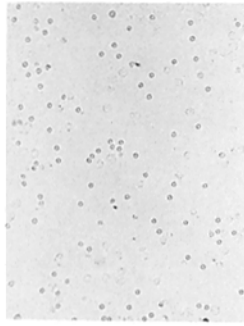
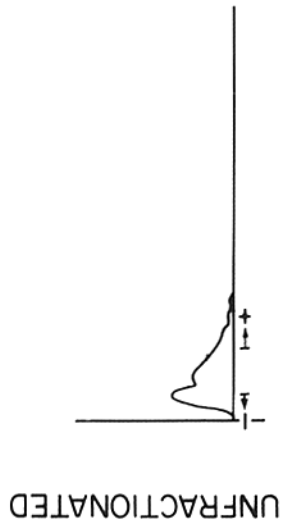


TABLE I
Outgrowth of Unseparated and BLAST-2 (EBVCS)⁺- and BLAST-2⁻ Populations

Plating density <i>Cells/ml</i>	Wells outgrown		
	Unseparated	BLAST-2 ⁺	BLAST-2 ⁻
5×10^5	6	0	6
2×10^5	6	0	6
1×10^5	2	0	6
5×10^4	0	0	6
2×10^4	0	0	6
1×10^4	0	0	6

The cell populations used were the same as described in the legend to Fig. 3. 0.2 ml of cell suspension at the given cell density were plated out in flat-bottomed microtiter plates and assessed visually for growth transformation at 10 d. Six wells were tested per group.

cells/ml. By comparison, outgrowth of unfractionated cells was seen at as low as 10^5 cells/ml, with one third of the wells being BLAST-2⁺, whereas outgrowth of the positively selected population was seen in all wells at the lowest cell number tested (10^4 cells/ml).

We wished to obtain an estimate of the fraction of BLAST-2 (EBVCS)⁺ cells that would undergo blastogenesis and outgrowth. To do this, the cloning efficiency of these cells was compared to that of a recently established cell line. Cells at 36 h postinfection were sterilely sorted, as described above, and cloned, using a Coulter autocloner, onto microtiter plates at a cell number of 100, 10, or 1 cell/well on a human fibroblast feeder layer. As a control, the same cells were also cloned at 14 d postinfection, by which time all of the cells are growth transformed. The sort parameters are given in the legend to Table II. In Table II, the BLAST-2 (EBVCS)⁺ cells had a preblastogenesis cloning efficiency (1.7%, or 1.9% when corrected for fraction of BLAST-2⁺ cells) similar to the established line (1.6%). Moreover, when the cloning efficiency for the unfractionated population at 36 h (0.27%) was corrected for the fraction of cells expressing BLAST-2 (EBVCS), a cloning efficiency (1.8%) similar to that of the established line was again obtained. No outgrowth was seen in any wells containing BLAST-2 (EBVCS)⁻ cells.

Thus, we may conclude that the BLAST-2 (EBVCS)⁺ cells at 36 h postinfection clone with a similar efficiency to that of established EBV-growth-transformed cells. This is consistent with all of the BLAST-2 (EBVCS)⁺ cells being activated, and undergoing blastogenesis and subsequent proliferation.

FIGURE 3. Capacity of the BLAST-2 (EBVCS)⁺ and BLAST-2⁻ populations to undergo subsequent transformation. 10^5 PBL-B cells were infected at 4×10^6 cells/ml with undiluted supernatant from the B95-8 lymphoblastoid cell line. 36 h postinfection, the cells were stained with anti-BLAST-2 (EBVCS) and analyzed with a FACS IV cell sorter. The 20% most and least fluorescent populations (*top*) were then sterilely separated. Reanalyses of the two populations are presented in the middle and lower panels. The 20% level was selected by comparison to the staining obtained with a negative control antibody, so as to give the highest yield of BLAST-2 (EBVCS)⁺ cells with the lowest level of contamination with BLAST-2 (EBVCS)⁻ cells. In the reanalyses, <1% of the negatively selected, and 60% of the positively selected cells expressed BLAST-2 (EBVCS). The cells were then placed back in culture and assessed daily for morphological transformation.

TABLE II
Cloning Efficiency of BLAST-2 (EBVCS)⁺, BLAST-2⁻, and Unseparated Populations Before Transformation Compared to the Established Cell Line 14 d Postinfection

Cells/Well	Cloning efficiency after 36 h in culture			Cloning efficiency of established line 14 d in culture
	Unseparated	BLAST-2 ⁺	BLAST-2 ⁻	
1	0	0	0	0
10	3/96	16/96	0	14/86
100	26/96	43/43	0	96/96
Cloning efficiency:	0.27	1.7	<0.01	1.6 [†]
Cloning efficiency (per cent per BLAST-2 ⁺ cell):	1.8	1.9		1.6

In this experiment, the unseparated culture contained 15% BLAST-2 (EBVCS)⁺ cells. The positive and negative populations were obtained by selecting the 10% most and least fluorescent cells. Upon reanalysis, the positive population contained 89% and the negative populations contained <1% BLAST-2 (EBVCS)⁺ cells. Cloning efficiency was estimated after 6 wk in culture.

Size and DNA Content of the BLAST-2 (EBVCS)⁺ Cells. If the BLAST-2 (EBVCS)⁺ cells that are detected on day 1 and day 2 postinfection have yet to undergo blastogenesis, they should have levels of DNA synthesis and size characteristic of resting PBL-B, and not of proliferating lymphoblasts. Our initial analysis, of the kind shown in Fig. 2, suggests that this was indeed the case. To test this directly, the BLAST-2 (EBVCS)⁺ cells were analyzed 36 h postinfection for size, and number of dividing cells. Using a FACS analyzer, it was possible to gate only on the BLAST-2 (EBVCS)⁺ population, and generate a size distribution curve. As may be seen in Fig. 4, this cell population had both a peak and mean cell volume indistinguishable from that obtained with purified uninfected PBL-B cells, but clearly distinct from that obtained with EBV-growth-transformed lymphoblasts. Similarly, when this same population was analyzed for DNA content using the propidium iodide stain (Fig. 4), essentially all of the cells were diploid, and, therefore, in the G₀-G₁ phase of the cell cycle, as was seen with the uninfected PBL-B cells. By comparison, more than half of the cells in the lymphoblastoid cell line were in the S-G₂-M phases of the cell cycle, characteristic of actively growing cells.

We conclude, therefore, that the BLAST-2 (EBVCS) antigen is expressed on a population of small resting B lymphocytes before blastogenesis.

RNA Content of the BLAST-2 (EBVCS)⁺ Cells. Another parameter for assessing the activation state of EBV-infected cells is the level of RNA synthesis. To assess relative RNA content, the small EBV-infected B cells were stained for RNA content at 36 h postinfection using the Hoechst 33342 and Pyronin Y double-staining method (22). As may be seen in Fig. 5C the infected B cells contain two distinct populations, one with a level of RNA similar to that of resting B cells (Fig. 5A); and one, constituting 42% of the cells, with a higher level of RNA, similar to that of proliferating lymphoblasts (Fig. 5B). When the BLAST-2 (EBVCS)⁺ cells were analyzed for RNA content, it was found (Fig. 5D) that they all expressed high levels of RNA, consistent with all being activated.

EBV Genetic Information in the BLAST-2 (EBVCS)⁺ Population. In the experiment described above, there was a marked discrepancy between the percentage

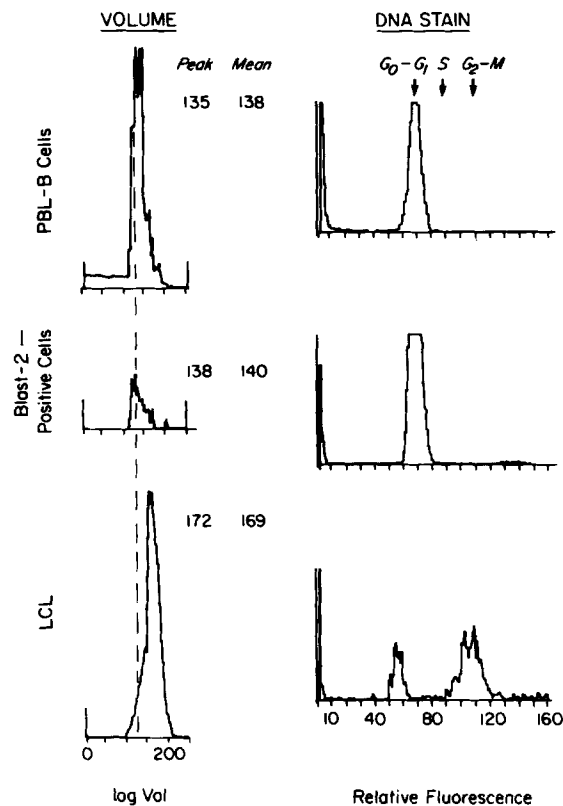


FIGURE 4. Size and cell cycle stage of the BLAST-2 (EBVCS)⁺ cells 36 h postinfection. Infected PBL-B cells were prepared and labelled as described for Fig. 1. In addition, uninfected PBL-B cells and an EBV-transformed lymphoblastoid cell line were studied. (*Left*) Using a FACS analyzer, the population of cells fluorescently labelled with anti-BLAST-2 (EBVCS) were gated, then analyzed for their size distribution in comparison to uninfected PBL-B cells or a transformed lymphoblastoid cell line. (*Right*) As above, except the cells were double-labelled with the DNA stain propidium iodide, and the BLAST-2 (EBVCS)⁺ cells were assessed for their cell cycle stage on the basis of their degree of labelling with propidium iodide compared to resting, uninfected PBL-B cells and an actively proliferating lymphoblastoid cell line.

of activated cells as assessed by BLAST-2 (EBVCS) expression (12%) compared to elevated RNA content (42%). However, the cloning experiments described above indicated that only the BLAST-2 (EBVCS)⁺ cells became immortalized. An alternative approach to assessing whether EBV had infected and thereby activated the B cells was to stain them for EBNA. Table III summarizes data from two experiments. Essentially all of the BLAST-2 (EBVCS)⁺ cells were also EBNA⁺, consistent with the results of the outgrowth experiment. However, most of the RNA-active cells were also EBNA⁺, indicating that there was a substantial fraction of cells that are EBNA⁺ and RNA active that did not express detectable levels of BLAST-2 (EBVCS). Furthermore, these cells do not grow out into established cell lines, as the cloning experiment indicates no detectable outgrowth with BLAST-2 (EBVCS)⁻ cells. We may conclude, therefore, that in an infected culture, at 36 h there are three types of small nondividing cells: (a) those that lack detectable BLAST-2 (EBVCS), do not express EBNA, and are not synthe-

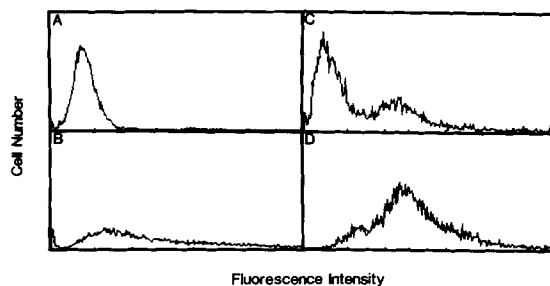


FIGURE 5. RNA content of BLAST-2 (EBVCS)⁺ cells. 36 h postinfection, PBL-B cells were infected with EBV and cultured for 36 h. The cells were then stained for BLAST-2 (EBVCS) expression and for RNA using the method described by Shapiro (22). (A) Resting uninfected PBL-B cells. (B) Recently established lymphoblastoid cell line. (C) Whole infected PBL-B cells. (D) BLAST-2 (EBVCS)⁺ infected PBL-B cells. There were 12% BLAST-2 (EBVCS)⁺ cells in the whole infected PBL-B cell population in this experiment.

TABLE III
Expression of EBNA and Elevated Levels of RNA in BLAST-2 (EBVCS)⁺ and BLAST-2⁻ Populations

	Total cells	EBNA ⁺ cells	RNA elevated %	Out- growth*
BLAST-2 ⁺	12	90	89.9	+
RNA Elevated	42	87		
EBNA ⁺	45			
BLAST-2 ⁻	88	38 [‡]	33.7	-
RNA Resting	58	10		
EBNA ⁻	55			

* Taken from experiments shown in Tables I and II.

[‡] Estimated from the percent EBNA⁺ cells in the total population, the percent of BLAST-2 (EBVCS)⁺ cells and the percent EBNA⁺ cells in that population.

sizing RNA (45–55%; Table III); (b) those that lack detectable BLAST-2 (EBVCS), but do express EBNA, and are actively synthesizing RNA (30–40%; Table III); and (c) those that do express detectable BLAST-2 (EBVCS) and EBNA, and are synthesizing RNA (10–15%; Table III). This last population completes the activation process and becomes a line of established proliferating lymphoblasts.

EBV Genome in the BLAST-2 (EBVCS)⁺ and BLAST-2⁻ Populations. If the BLAST-2 (EBVCS)⁺ PBL-B cells that occur after infection are destined to undergo blastogenesis and proliferate into continuously growing lymphoblastoid cell lines, they should contain EBV genomes, in addition to expressing EBNA. To test this prediction, the BLAST-2 (EBVCS)⁺ and BLAST-2⁻ populations were separated and collected as described above, except that a FACS IV was used. ~10⁶ cells of each population and of the unfractionated cell population were then blotted onto nitrocellulose paper. RAJI cells, an EBV⁺ Burkitt's lymphoma line with ~60 intact viral genomes (28), and RAMOS cells, an EBV⁻ Burkitt's lymphoma line (29) were blotted as controls. The presence of EBV DNA was then detected by probing the blots with the Bam HI V fragment of the EBV genome. The autoradiogram obtained from the hybridized filter is

shown in Fig. 6. This result demonstrates qualitatively that EBV genetic information was readily found in the unfractionated and BLAST-2 (EBVCS)⁺ populations. Furthermore, the hybridization signal was enriched after isolation of the BLAST-2 (EBVCS)⁺ cells with an intensity on the autoradiogram suggestive of multiple EBV genome copies. More surprisingly, we were unable to detect any EBV DNA in the BLAST-2 (EBVCS)⁻ population (see discussion). No hybridization was seen to the negative control RAMOS cell line, and a strong signal was obtained with the positive control RAJI cell line. These results lead to the conclusion that the BLAST-2 (EBVCS)⁺ population already contains multiple copies of the EBV genome.

Expression of BLAST-2 (EBVCS) in the Presence of Metabolic Inhibition. As shown above, the expression of BLAST-2 (EBVCS) upon EBV infection occurs before detectable cellular DNA synthesis or cell division, but concomitant with raised levels of RNA. To define whether the appearance of BLAST-2 (EBVCS) was a consequence of de novo synthesis, or exposure of cryptic antigen, we attempted to block the expression with various metabolic inhibitors. As shown in Table IV, the presence of the DNA synthesis inhibitor arabinoside c did not affect the expression of BLAST-2 (EBVCS). However, BLAST-2 (EBVCS) expression was prevented by a protein synthesis inhibitor (cycloheximide), and an inhibitor of RNA synthesis (actinomycin D). These experiments suggest, therefore, that the gene encoding BLAST-2 (EBVCS) may be activated upon EBV infection, rather than EBV infection causing the surface expression of BLAST-2 (EBVCS) molecules already present.

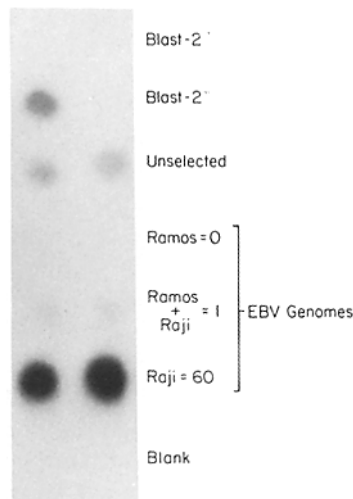


FIGURE 6. Detection of EBV genetic information in the BLAST-2⁺ and BLAST-2⁻ populations. BLAST-2 (EBVCS)⁻ and BLAST-2⁺ cell populations were obtained by sorting infected PBL-B cells at 36 h postinfection, as described in Fig. 4. 10^6 cells of each population were then blotted onto nitrocellulose, along with the unselected population. The cell lines RAMOS and RAJI were used as negative and positive controls, respectively. In addition, a mixture of RAMOS and RAJI, which should yield an average genome number around 1, was also applied. All cell types are present in duplicate, with the exception of the sorted (BLAST-2 (EBVCS)⁻ and BLAST-2⁺) populations, for which there is only one spot each. The blots were then processed and hybridized with the Bam H1 V fragment of the EBV genome by standard techniques (see Materials and Methods).

TABLE IV
Effect of Metabolic Inhibitors on Expression of BLAST-2 (EBVCS)

Inhibitor	BLAST-2 ⁺ Cells	Inhibition
Arabinoside C (1 µg/ml)	12.1	7
Control*	13.0	
Actinomycin D (1 µg/ml)	1.1	93
Control*	16.4	
Cycloheximide (5 µg/ml)	2.4	75
Control*	9.7	

* These represent three separate experiments performed at different times, therefore, the control value for each is included. The control represents the same cells infected in the absence of inhibitor.

Discussion

In this report, we show that the *in vitro* infection of B cells by EBV may be used to delineate events occurring during B cell activation, and conversely, that surface markers of B cell differentiation may be used as probes for analyzing the early events in EBV infection. In our previous studies (13) we have shown that the BLAST-2 (EBVCS) antigen appears before blastogenesis on B cells that have been activated by a variety of agents. However, it was not possible to conclude that these cells were destined to undergo blastogenesis. Using the EBV system, we have now shown this to be true. Furthermore, we suggest that this is typical for activation of B cells by any other agent. We have isolated the BLAST-2 (EBVCS)⁺ population early in infection, and demonstrated that it consists of small diploid B cells, clearly distinct from proliferating lymphoblasts. These cells contain high levels of RNA, multiple copies of the viral genome, and express the viral genetic information (EBNA). Furthermore, they clone with a frequency similar to that of a recently established EBV-infected cell line. By comparison, the BLAST-2 (EBVCS)⁻ population did not immortalize or contain detectable viral genomes, although a large fraction of the cells expressed EBNA and elevated levels of RNA. The observation that a substantial number of EBV-infected EBNA⁺ cells do not proliferate is in agreement with similar conclusions drawn by Robinson and Smith (18), based on kinetic studies. The presence of EBNA in some of the BLAST-2 (EBVCS)⁻ cells implies the presence of viral genomes at a low copy number. This would have to be of the order of one genome per cell in order to remain undetectable in our experiment. This number is considerably lower than the average copy number in the BLAST-2 (EBVCS)⁺ population. We conclude, therefore, that BLAST-2 (EBVCS) is the earliest B cell-specific marker for activated cells, and defines a stage equivalent to entry into an early G₁. Furthermore, it is a reliable indicator for immortalization by EBV. The blocking of antigen expression by inhibitors of RNA and protein synthesis implies that the antigen may be newly synthesized rather than cryptic.

The stage of activation defined by BLAST-2 (EBVCS) has some features in common with early G₁, as described by others (1, 2). These authors have described a stage that occurs soon after the activating signal, and is characterized by increased RNA synthesis in nondividing cells. However, these authors also describe these cells as having increased size, thus distinguishing them from the still small BLAST-2 (EBVCS)⁺ cells.

We have noted (13) that anti-BLAST-2 (EBVCS) gave an intense reaction in stains of histologic cross sections of lymph nodes. This stain was restricted to the germinal centers, as is expected for a B cell-specific antigen that appears on lymphoblasts. However, stain was also seen associated with an occasional cell in the mantle zone, which contains small B cells. We were able to exclude the possibilities that this reaction was due to the presence of a few B lymphoblasts or a small population of BLAST-2 (EBVCS)⁺ PBL-B cells. On the basis of the present study, we may hypothesize that the BLAST-2 (EBVCS)⁺ cells in the mantle zone may represent antigen-activated B cells that have not yet migrated into the germinal center and undergone blastogenesis.

Chronic lymphocyte leukemia (CLL) of B cell origin was thought to arise from a cell equivalent to the small circulating peripheral B cell; nevertheless, we have shown previously (12, 13) that these cells express the BLAST antigens. We suggest, therefore, that the B CLL may be equivalent to the activated peripheral B cell that has not yet undergone blast transformation, but would still express the BLAST-2 (EBVCS) antigen.

The studies discussed above indicate that the EBV system can be used to study events in B cell activation, and that this information may then be used to interpret findings obtained with other more immunologically relevant systems. Conversely, we are currently using the BLAST antigens as probes for studying the virology of the early events in EBV infection.

The conversion of normal cells into tumor cells has been shown in many cases to be due to the inappropriate expression of cellular genes (oncogenes) or their viral counterparts. It remains to be seen whether the inappropriate level of expression of the BLAST-1 and BLAST-2 (EBVCS) antigens during EBV infection plays a direct role in the immortalization process, in addition to their presumed role in transformation/activation. The alternative explanation is that the appearance of BLAST-1 and BLAST-2 (EBVCS), being concomitant with activation, occurs automatically when B cells move from a resting state to the lymphoblastoid stage. The aberrant levels of the antigens in an autonomously replicating, EBV-infected cell would then simply reflect loss of control of gene expression during the immortalization process.

In conclusion, we have described two B cell-specific antigens that define different stages of activation of human B cells. In this paper, we have shown that BLAST-2 (EBVCS) appears on the resting cell soon after the activation signal. This cell then proceeds to undergo blast transformation which, we have shown previously (12), is associated with the expression of the BLAST-1 antigen. Both of the antigens are expressed at aberrantly high levels only on EBV-infected cells. It is likely that these two molecules play important roles both in the mechanism of normal B cell activation, and of EBV-induced immortalization.

Summary

We have used Epstein-Barr virus (EBV) infection *in vitro* to delineate two distinct stages in B cell activation. Previous studies have shown that the BLAST-2 (EBVCS) (EBV cell surface) activation antigen is expressed on a small fraction of B cells within 24 h of stimulation with a variety of agents, including mitogens and EBV. In this study, we have been able to isolate the BLAST-2 (EBVCS)⁺ cells early after activation/infection with EBV. These cells are small B cells that

are actively synthesizing RNA but not DNA, and are, therefore, clearly distinct from large proliferating lymphoblasts. In addition, they contain multiple copies of the EBV genome, express the viral nuclear antigen (EBNA) and, most importantly, proceed to undergo transformation when placed back in culture. By comparison, the BLAST-2 (EBVCS)⁻ population does not undergo transformation, even though a fraction of these cells are activated for RNA synthesis and express EBNA. Thus, using the EBV system, we have been able to show directly that an activated B cell first expresses the BLAST-2 (EBVCS) antigen concomitant with an increase in RNA synthesis, and then subsequently proceeds to differentiate into a proliferating lymphoblast.

The authors would like to thank Yvonne Jones for typing this manuscript.

Received for publication 19 February 1985.

References

1. Darzynkiewicz, Z., F. Traganos, and M. R. Melamed. 1980. New cell cycle compartments identified by multiparameter flow cytometry. *Cytometry*. 1:98.
2. Monroe, J. G., and J. C. Cambier. 1983. Sorting of B lymphoblasts based upon cell diameter provides cell populations enriched in different stages of cell cycle. *J. Immunol. Methods*. 63:45.
3. Kehrl, J. H., A. Muraguchi, and A. S. Fauci. 1984. Differential expression of cell activation markers after stimulation of resting human B lymphocytes. *J. Immunol.* 132:2857.
4. Schneider, U., and H. zur Hausen. 1975. Epstein-Barr virus-induced transformation of human leukocytes after cell fractionation. *Int. J. Cancer*. 15:59.
5. Yata, J., C. Desgranges, T. Nakagawa, M. C. Favre, and G. de-The. 1975. Lymphoblastoid transformation and kinetics of appearance of viral nuclear antigen (EBNA) in cord-blood lymphocytes infected by Epstein-Barr virus (EBV). *Int. J. Cancer*. 15:377.
6. Menezes, J., M. Jondal, W. Leibold, and G. Dorval. 1976. Epstein-Barr virus interactions with human lymphocyte subpopulations: Virus adsorptions, kinetics of expression of Epstein-Barr virus-associated nuclear antigen, and lymphocyte transformation. *Infect. Immun.* 13:303.
7. Thorley-Lawson, D. A., and J. L. Strominger. 1978. Reversible inhibition by phosphonoacetic acid of human B lymphocyte transformation by EBV. *Virology* 86:423.
8. Henderson, E., G. Miller, J. Robinson, and L. Hedan. 1977. Efficiency of transformation of lymphocytes by EBV. *Virology*. 76:154.
9. Åman, P., B. Ehlin-Henriksson, and G. Klein. 1984. Epstein-Barr virus susceptibility of normal human B lymphocyte populations. *J. Exp. Med.* 159:208.
10. Rosen, A., P. Gergely, M. Jondal, G. Klein, and S. Britton. 1977. Polyclonal Ig production after Epstein-Barr virus infection of human lymphocytes in vitro. *Nature (Lond.)*. 267:52.
11. Bird, A. G., and S. Britton. 1979. A new approach to the study of human B lymphocyte function using an indirect plaque assay and a direct B cell activator. *Immunol. Rev.* 45:41.
12. Thorley-Lawson, D. A., R. T. Schooley, A. K. Bhan, and L. M. Nadler. 1982. Epstein-Barr virus superinduces a new human B cell differentiation antigen (BLAST-1) expressed on transformed lymphoblasts. *Cell*. 30:415.
13. Thorley-Lawson, A. D., L. M. Nadler, A. K. Bhan, and R. T. Schooley. 1985.

- BLAST-2 (EBVCS), An early cell surface marker of human B cell activation, is super-induced by Epstein Barr virus. *J. Immunol.* 134:3007.
14. Henle, W., V. Diehl, G. Kohn, H. zur Hausen, and G. Henle. 1967. Herpes-type virus and chromosome marker in normal leukocytes after growth with irradiated Burkitt cells. *Science (Wash. DC)*. 157:1064.
 15. Miller, G., J. F. Enders, and H. I. Lisco. 1969 Establishment of lines from normal human blood leukocytes by co-cultivation with a leukocyte line derived from a leukemic child. *Proc. Soc. Exp. Biol. Med.* 132:247.
 16. Robinson, J., and D. Smith. 1981. Infection of human B lymphocytes with high multiplicities of Epstein-Barr virus: Kinetics of EBNA expression, cellular DNA synthesis, and mitosis. *Virology*. 10:336.
 17. Hennessy, K., S. Fennewald, M. Hummel, T. Cole, and Kieff. 1984. A membrane protein encoded by Epstein-Barr virus in latent growth-transforming infection. *Proc. Natl. Acad. Sci. USA*. 81:7207.
 18. Kinter, C., and B. Sugden. 1981. Identification of antigenic determinants unique to the surfaces of cells transformed by Epstein-Barr virus. *Nature (Lond.)*. 29:458.
 19. Chess, L., and S. F. Schlossman. 1976. Methods for the separation of unique human lymphocyte subpopulations. In *Manual of Clinical Immunology*. R. Rose and H. Friedman, editors. American Society for Microbiology. Washington, D. C. 77.
 20. Thorley-Lawson, D. A., L. Chess, and J. L. Strominger. 1977. Suppression of in vitro Epstein-Barr virus infection. A new role for adult human T lymphocytes. *J. Exp. Med.* 146:495.
 21. Stashenko, P., L. M. Nadler, R. Hardy, and S. F. Schlossman. 1980. Characterization of a human B lymphocyte specific antigen. *J. Immunol.* 125:1678.
 22. Shapiro, H. M. 1981. Flow cytometric estimation of DNA and RNA content in intact cells stained with Hoechst 33342 and Pyronin Y. *Cytometry*. 2:143.
 23. Grunstein, M., and D. Hogness. 1975. Colony hybridization: A method for the isolation of cloned DNA's that contain a specific gene. *Proc. Natl. Acad. Sci. USA*. 72:3961.
 24. Brandsma, J., and G. Miller. 1980. Nucleic acid spot hybridization: Rapid quantitative screening of lymphoid cell lines for Epstein-Barr viral DNA. *Proc. Natl. Acad. Sci. USA*. 77:6851.
 25. Reedman, B. M., and G. Klein. 1973. Cellular localization of an Epstein-Barr virus (EBV)-associated complement fixing antigen in producer and non-producer lymphoblastoid cell lines. *Int. J. Cancer*. 11:499.
 26. Einhorn, L., and I. Ernberg. 1978. Induction of EBNA precedes the first cellular S-phase after EBV-induction of human lymphocytes. *Int. J. Cancer*. 21:157.
 27. Todd, R. F., III, L. M. Nadler, and S. F. Schlossman. 1981. Antigens on human monocytes identified by monoclonal antibodies. *J. Immunol.* 126:1435.
 28. Adams, A. 1979. The virus Genome and Host cell DNA, in the Epstein-Barr Virus. M. A. Epstein and B. G. Achong, editors. Springer-Verlag, New York. p. 158.
 29. Anderson, M., and T. Lindahl. 1976. Epstein-Barr virus DNA in human lymphoid cell lines: in vitro conversion. *Virology*. 73:96.