CHARACTERISTICS OF EPSTEIN-BARR VIRUS ACTIVATION OF HUMAN B LYMPHOCYTES*

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Epstein-Barr virus (EBV)[1] is a herpes virus with a very narrow tropism in that it can only infect humans and primates, and only the B lymphocytes of these susceptible species (1). The virus will activate the host cell from a state of rest to a high rate of immunoglobulin (Ig) secretion (2) and, moreover, a proportion of the infected B cells have been shown to proliferate indefinitely in vitro (3). This virus is the causative agent in infectious mononucleosis (4), and it has strong association with certain human B lymphocyte tumors (1).

Of these multiple aspects of EBV, we have focused on its capacity to induce Ig secretion in resting B cells from human peripheral blood. Because EBV appears to be the only agent so far fully evaluated that can activate B cells to Ig secretion in the absence of other cellular influences (5), we assume that it will be used as an analytical tool in assessing human B lymphocyte function in future clinical practice. The cellular mechanisms of the activation and range of infectivity for B lymphocytes therefore needs to be established. We also speculate that EBV will prove important in depicting the subcellular processes preceding and accompanying activation, because EBV can be traced intracellularly by virtue of the sequence of antigens it generates. The development of the host-virus relationship might be correlated with the metabolic steps involved in moving the B cell from rest to a high rate of Ig secretion.

In this communication, we demonstrate that there is a definite correlation between the induction of viral antigens, de novo synthesis of cellular DNA, and Ig secretion of in vitro EBV-infected B cells. Not all B cells are infectible by EBV, however, and a smaller fraction of the B cells can both be infected and activated to Ig secretion by EBV.

Materials and Methods

Cell Preparations. Mononuclear leukocytes were obtained from the blood of adult healthy donors. They were separated from the buffy coat of citrate blood on Ficoll-Isopaque gradients (6).

Purified Lymphocyte Populations. These were of two kinds. First, T and B cells were fractionated by erythrocyte rosette separation. Lymphocytes binding sheep erythrocytes (SRBC) were spun

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[1] *Abbreviations used in this paper:* Ara-C, cytosine arabinoside; EBNA, Epstein-Barr nuclear antigen; EBV, Epstein-Barr virus; FBS, fetal bovine serum; PFC, plaque-forming cells; PWM, pokeweed mitogen; SRBC, sheep erythrocytes.

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on a Ficoll gradient, and the layer not binding SRBC was recovered. These cells (B1) contained <5% SRBC binding lymphocytes as judged by rerosetting, but contained >50% monocytes as judged by acridin orange staining (7).

The extensively purified B cell preparation (B2) was obtained by first clearing the Ficoll-separated cells from monocytes by exposing them to iron powder, followed by magnet treatment. Then the cells were passed through a nylon wool column and the retained cells were eluted by fetal bovine serum (FBS) at 37°C. The eluted fraction was rosetted with SRBC and spun over Ficoll-Isopaque to remove any remaining SRBC-binding T cells. This yielded a cell suspension with <1% SRBC binding cells and no proliferative response to T cell mitogens (8).

Tissue Culture. The cells were cultivated either in 2-ml round-bottomed tubes or 25-ml flasks in 5% CO_2 in air. The RPMI 1640 medium was supplemented with either 10% heat-inactivated FBS or human AB serum and antibiotics (penicillin and gentamycin).

Viral Exposure of Cells. Two preparations of EBV were used, i.e., the transforming virus obtained from the supernate of the B95-8 marmoset line (9) and the nontransforming variant from the supernate of the P3HR-1 Burkitt-derived line (10). Both virus-containing supernates were filtered (0.45-μ pore size) before use.

Virus exposure was performed by suspending the cell pellets in virus-containing supernate and incubating this mixture for 60 min at 37°C at a concentration of 10^6 cells/ml. After this incubation, the cells were washed twice and cultivated in serum-supplemented RPMI 1640. When exposed to the T cell-dependent activator pokeweed mitogen (PWM) (Techtum, Umeå, Sweden), the cells were in continuous contact with the agent at a concentration of 20 μg/ml.

Measurement and Inhibition of DNA Synthesis. Triplicate cultures of 200 μl were cultured in microculture plates (3040; Falcon Labware, Div. of Becton Dickinson & Co., Oxnard, Calif.). 1 μCi [^{3}H]thymidine was added and the cells were incubated for an additional 6-12 h and then harvested in a microharvester. The dried filters were counted in a liquid scintillation counter. Inhibition of DNA synthesis was obtained by exposing the cells to cytosine arabinoside (Ara-C) (Sigma Chemical Co., St. Louis, Mo.) at 20 μg/ml for 36 h.

Demonstration of EBV-determined Nuclear Antigen (EBNA). Methanol-acetone-fixed smears of cells were prepared and stained by anti-complement immunofluorescence (11) after exposure to an anti-EBNA-positive serum.

Protein-A Plaque Assay. This was performed as we have described previously (5). Protein-A-coupled erythrocytes were obtained by exposing one part washed and packed SRBC to one part staphylococcal protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) at a concentration of 0.5 mg/ml in 10 parts $CrCl_3$. Rabbit anti-human μ, γ, and α antisera (Dako Ig, Copenhagen, Denmark), diluted 1:50, were used as developing sera. Guinea pig serum absorbed with equal volumes of packed SRBC for 1 h at 4°C was the source of complement. Plaque-forming cells (PFC) are expressed as PFC/10^6 cells initially cultured.

Results

Relationship between Ig Production, De Novo DNA Synthesis, and Intracellular Presence of Viral Antigens in B Lymphocytes Exposed to EBV. Highly purified B cells (B2) were exposed to dilutions of B95-8 supernate. In such treated cells, the proportion of EBNA-positive cells was assayed after 2 and 6 d, respectively, and the number of IgM-producing B cells was measured, as well as the level of DNA synthesis after 7 d of culture (Fig. 1). There is a linear decrease of all these parameters as a function of virus dilution.

In Fig. 2, it is evident that the induction of IgM-secreting cells falls linearly as a function of virus dilution in both purified (B1) and nonpurified (B plus T) lymphocyte preparations. The purified B cells, however, yield higher peak values and support a response with more dilute virus preparations.

Role of De Novo DNA Synthesis and Transformation in the EBV-dependent Induction of Ig Secretion. We sought to establish the requirement for cell division in the activation of B cells from a state of rest to a high rate of Ig secretion by performing the EBV

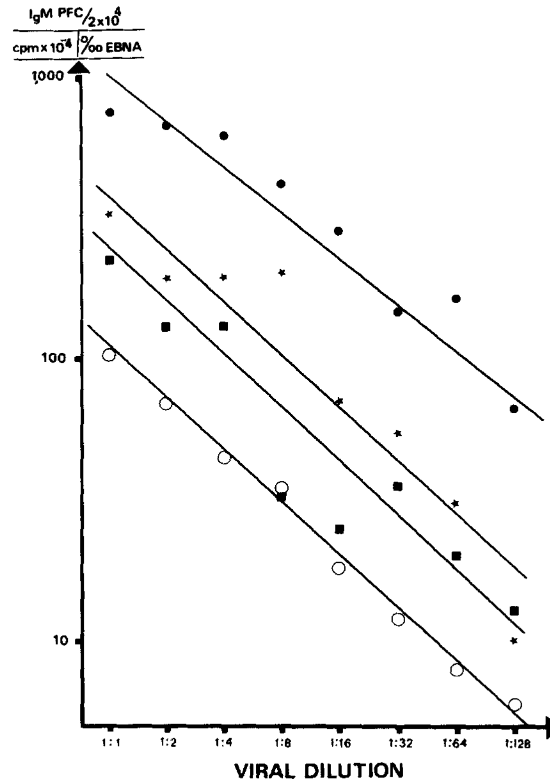

FIG. 1. Effect of viral dilution on appearance of EBNA-containing cells on day 2 (○) and day 7 (★); total IgM-secreting cells (■), and DNA synthesis on day 7 (●) in extensively purified B cells (B2).

exposure of highly purified B lymphocytes (B2) in the presence of Ara-C (12). Table I shows that cells that have been in contact with EBV (B95-8) for 7 d, but were in the presence of Ara-C for the last 36 h, show a marked reduction of DNA synthesis and also generate fewer Ig-secreting cells than the controls without Ara-C (Table I). Ara-C was also added 36 h after virus exposure and the EBNA staining and determination of Ig-secreting cells was performed 36 h later, i.e., 3 d after starting the culture. No Ig-secreting cells were discernable in this experiment, although 13% of the cells stained for EBNA, which would indicate the proportion of cells infectible by EBV in this preparation. In other preparations, where DNA synthesis was inhibited by Ara-C at this early stage, up to 30% of the purified B cells have been infectible by EBV as judged by EBNA positivity.

An alternative approach for establishing the requirement for B cell transformation in the maturation leading to active Ig secretion was to expose the cells to the nontransforming variant of EBV, i.e., P3HR1. This virus preparation failed (Table II) to induce Ig secretion, as well as DNA synthesis in human B cells, but it could specifically block these actions from subsequently added transforming EBV (B95-8). This effect of P3HR1 is not the result of a toxic or cytostatic action of the virus upon B cells in general, because such treated cells can still be activated by PWM (Table II).

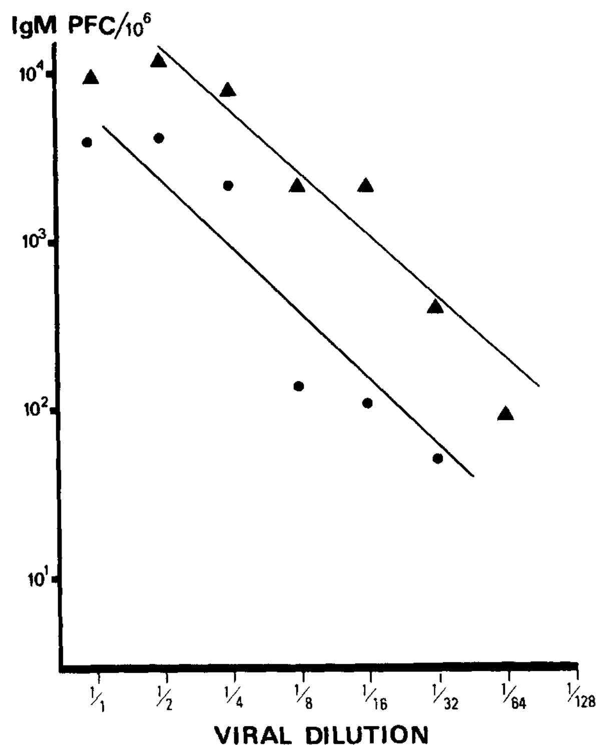

FIG. 2. Effect of viral dilution on the total number of IgM-secreting cells on day 7 in purified B (B1) cells (▲), and unseparated peripheral blood lymphocytes (●).

TABLE I

Number of IgM, IgG, and IgA PFC, the Content of EBNA-positive Cells, and the Level of DNA Synthesis in Extensively Purified B Cells (B2) Exposed to EBV (B95-8) in the Presence or Absence of Ara-C, and Tested after 7 d in Culture

Cell material	DNA synthesis level of [^{3}H]thymidine per 10^6 cells*	IgM PFC/10^6	IgA PFC/10^6	IgG PFC/10^6	Percent EBNA-positive cells at day 7
B2 cells alone	2,700	300	150	0	0
B2 + Ara-C‡	2,750	0	50	50	0
B2 + EBV§	21,690	7,000	1,550	2,250	54
B2 + EBV + Ara-C	650	2,000	100	900	36

* DNA synthesis was measured by exposing the cells to 1 μCi [^{3}H]thymidine at the end of culture (day 7) and incubating for an additional 12 h.

‡ 20 μg Ara-C was added 36 h before harvest at day 7.

§ Cells were exposed to the supernate of B95-8 line (10^6/ml) for 1 h. Thereafter they were washed and cultivated in RPMI 1640 with 10% FBS in round tubes.

Possible Role of T Cells in Stopping the Development of Ig-secreting Cells in Short-Term Culture Stimulated by EBV. In comparing the EBV responsiveness of purified (B1) vs. nonpurified (B plus T) B cells, we also studied the kinetics of response measured by the development of IgM-secreting cells. T cell-deprived preparations showed a continuous increase of cells producing IgM, as well as a high proportion of EBNA-positive

TABLE II

Number of IgM and IgG PFC and DNA Synthesis in Cultures Exposed to Nontransforming EBV (P3HR-1) vs. Transforming EBV (B95-8) and the Inhibitory Effect of P3HR-1 on Polyclonal Ig Induction

Material added	PFC/10^6 cultured cells		DNA synthesis [^{3}H]thymidine incorporation per culture at day 7
	IgM	IgG	
B95-8*	6,400	3,760	6,950
P3HR-1*	410	90	870
PWM‡	10,200	7,860	27,300
P3HR-1 + B95-8§	800	740	1,800
B95-8 + P3HR-1	4,040	2,950	4,710
P3HR-1 + PWM	10,830	9,430	18,660
B95-8 + PWM	12,700	9,060	20,740
0	60	0	640

* Virus preparations were added by suspending 10^6 cells in 1 ml of virus supernates for 60 min at 37°C. After washes, the cells were incubated in RPMI 1640 with 10% human AB serum.

‡ 20 μg/ml of PWM added at day 0.

§ Sequential addition of virus was done by first exposing the cells to one virus as described above, followed by washes, and then to the second virus supernate, followed by washes, after which the cells were put in culture.

cells as late as 14 d after initiating the culture (Fig. 3), at a time when cell mixtures containing T cells showed very few Ig-secreting cells, as well as a low content of EBNA-positive cells.

Discussion

We have determined by these experiments that the EBV-induced activation of human B lymphocytes is a dose-dependent, one-hit phenomenon, and that the maturational step involved in the process of activation requires de novo DNA synthesis.

Although EBV receptors appear to be present on virtually all B cells, as well as some null cells (13, 14), only a fraction of these B cells (10–30%) appears in our experiments to be infectible by the virus, as judged by the appearance of EBNA before DNA synthesis is initiated. We can say this provided that the concentration of virus is not the limiting factor. This does not appear to be the case, as we see a plateau effect with the lower viral dilutions (Fig. 2). However, using extensively concentrated virus, larger proportions of B cells (≤90%) have on occasion been infected (15). We do not know what characterizes the B cells to which the virus can gain intracellular access, nor do we know what signifies the smaller fraction of the B cells (1–3%) that is driven to Ig secretion by the virus. We can perform the latter calculation because we know from our previous work that all Ig-producing cells in a cell culture exposed to EBV contain the EBNA antigen (2).

It is well established that the EBV infection proceeds via the induction of EBNA (10 h), and later, (40 h) of DNA synthesis (16).

Differentiation from a rest state to active Ig synthesis in a B cell after EBV infection appears to be dependent on de novo DNA synthesis. This is true for all three major isotypes (Table I). The development of IgM-synthesizing cells appears somewhat less

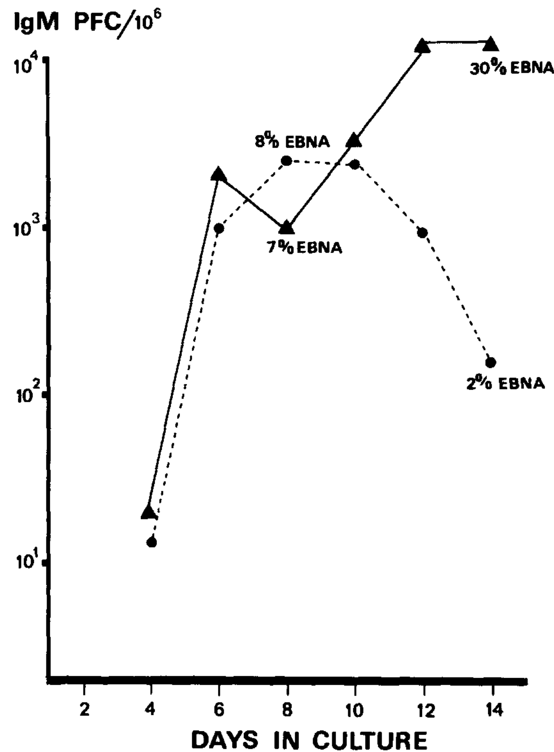

FIG. 3. Effects of T lymphocyte removal on the appearance of IgM-secreting B lymphocytes and EBNA-containing cells as a function of time after virus exposure. (●), unseparated peripheral blood lymphocytes; (▲), T cell-depleted (B1) blood lymphocytes. The cell donor was immune to EBV as determined by anti-virus capsid antigen titers.

sensitive to the action of Ara-C than to IgG and IgA. This is in agreement with previously published information (17), which indicates that an immunological switch from IgM to IgG is strictly dependent of DNA synthesis, whereas the path from rest to active IgM secretion seems to be less so. In this system, we know that the virus is incorporated in cellular DNA and that viral proteins are synthesized, although DNA synthesis is blocked, but that a high rate of Ig secretion is not initiated under those conditions. Recently, an agent has been described that can induce mainly IgM synthesis in human B cells without the need for de novo DNA synthesis (18). EBV and formalin-killed *Salmonella paratyphi* thus differ in this regard.

Nontransforming EBV, i.e., P3HR1, does not induce DNA synthesis or Ig formation in human B cells (Table II). Furthermore, P3HR1 will specifically block the Ig-inducing action of subsequently added transforming B95-8. This may suggest that the viruses compete for similar membrane and/or intracellular sites in the same cells. Because the Ig-inducing action of PWM is not blocked, one must conclude that PWM either activates different subsets of B cells or uses different pathways of activation (19).

Finally, T cell-deprived B cell suspensions do not clear themselves of the EBNA-positive cells, as the T cell-containing suspensions do (Fig. 3). These data support previously published evidence (20, 21) of T cell regulation of EBV-infected B cells.

Exact frequency analysis of responding cells will require limiting dilution analysis,

which this system does not lend itself to yet. Thus, we have tried to find out the frequency of eternalizable cells by doing single cell transfer of EBNA-positive PFC to microwells with fibroblast feeder cells. One interpretation of these experiments is that either these eternalizable B cells are not recruited from the high-rate Ig-producing cells or else they appear in low frequency (<1/50).

Summary

Epstein-Barr virus (EBV) will infect at least every third cell if exposed in vitro to an extensively purified B cell population from human peripheral blood. About 10% of such infected cells will be driven into immunoglobulin synthesis and secretion, as judged by the indirect protein A plaque assay. The appearance of EB nuclear antigen, de novo DNA synthesis, and immunoglobulin secretion are linked phenomena accompanying infection as judged by viral dilution experiments, which yield kinetics of a one-hit order.

Induction of immunoglobulin secretion in B cells by EBV requires de novo synthesis of DNA, and consequently, nontransforming EBV (P3HR1) will not induce immunoglobulin secretion and will also specifically block such induction from subsequently added EBV.

The termination of immunoglobulin induction by EBV in short-term cultures appears to be T cell dependent.

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