FREQUENCIES OF THE SEPARATE HUMAN B CELL SUBSETS ACTIVATABLE TO Ig SECRETION BY EPSTEIN-BARR VIRUS AND POKEWEED MITOGEN*

By OTONIEL MARTÍNEZ-MAZA[‡] AND SVEN BRITTON

From the Department of Immunobiology, Karolinska Institute, Wallenberglaboratory, S-104 05 Stockholm 50, Sweden

Epstein-Barr virus (EBV)[1] is a herpes-type virus that infects B lymphocytes in humans and other primates (1). It is the causative agent of infectious mononucleosis (2), and is associated with certain human B cell tumors, such as Burkitt's lymphoma (1, 3). EBV can polyclonally activate human B lymphocytes to immunoglobulin (Ig) secretion (4–6). This polyclonal activation, which is readily seen following the infection of human B cells by EBV in vitro, does not require the activity of either T cells or macrophages (5, 7). Therefore, EBV has shown great potential for use as a direct polyclonal B cell activator (PBA) for human B cells. Because of this, knowledge of the frequency of human B cells that can be activated to Ig secretion by EBV is not only of interest in understanding more about the interactions of EBV with human lymphocytes, but is essential in interpreting the results obtained when EBV is used as a PBA in studies on the immune potential of human B cells.

Here we present a quantification of the EBV-induced polyclonal activation of human B cells to Ig secretion. This was done by limiting dilution analysis of EBV-activated human B cells, to determine the precursor frequency (PF) of IgM and IgG-secreting cells. Limiting dilution analysis of murine B cells has been used to determine the frequency of B cells activatable by antigens or mitogens (8, 9). This technique has also been used to quantify the response of human B cells to pokeweed mitogen (PWM) (10).

In this report, the PF of IgM-secreting cells induced by EBV is compared to that induced by a commonly used, T cell-dependent PBA, PWM. Both EBV and PWM appeared to activate a small fraction of human B cells to IgM secretion, and these two PBA apparently acted on discrete B cell subpopulations.

Materials and Methods

Cell Preparation. Mononuclear leukocytes were separated from the blood of healthy adult donors by centrifugation of the buffy coat fraction of citrate blood, diluted 1:1 (vol/vol) with

[1] *Abbreviations used in this paper:* EBV, Epstein-Barr virus; Hepes, N-2-hydroxyethyl-piperazine-N′-2-ethanesulphonic acid; Ig, immunoglobulin; PBA, polyclonal B cell activator; PF, precursor frequency; PFC, plaque-forming cell; PWM, pokeweed mitogen; smIg+, surface membrane immunoglobulin positive; SRC, sheep erythrocyte.

sterile saline, over Lymphoprep (Nyegaard & Co., A/S, Oslo, Norway) gradients (11).

T and B Lymphocyte Separation. T and B cells were fractionated by erythrocyte rosette separation (12). Briefly, lymphocytes were allowed to form rosettes with sheep erythrocytes (SRC) by incubating at 37°C for 15 min, centrifuging at 200 g for 5 min at 20°C, then being kept at 4°C for 1–2 h. Rosette-forming cells were then separated from nonrosetted cells by centrifugation over Lymphoprep gradients (500 g for 25–30 min, at 20°C). B cell fractions (nonrosette-forming cells) contained <5% SRC-binding cells and ~50% surface membrane Ig positive (smIg+) cells, as measured by direct immunofluorescence (13–15). T cell fractions (SRC-rosetting cells) contained <10% nonrosette-forming cells and <7% smIg+ cells. When the T cell fractions were irradiated, this was done by exposing these cells to 2,000 R γ-radiation, before their use as filler cells. In experiments where nonirradiated T cells were used as filler cells, the T cell fraction was passed through nylon-wool columns two times before use (16).

Tissue Culture. Cells were cultured in 12 mm × 75 mm polystyrene test tubes (No. 2058, Falcon, Oxnard, CA), containing 0.2 ml of RPMI 1640 medium, supplemented with 10% heat-inactivated human AB serum, antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin), 10 mM Hepes buffer, and sodium bicarbonate (25 mM). Cultures were incubated in a humidified atmosphere, containing 5% CO_2, at 37°C for 7 d.

Viral Exposure of Cells. EBV-containing supernatant, from the B95-8 marmoset cell line (the kind gift of Dr. George Klein) (17), was used as the source of virus. This virus-containing supernatant was filtered (0.45-μm pore size) before use. Viral exposure was performed by suspending cells in the B95-8 supernatant at a concentration of 5×10^6 to 1×10^7 cells per ml of supernatant, and incubating for 1 h at 37°C (5–7). After exposure, the cells were washed two times and placed in the final culture medium at an appropriate cellular concentration. Cells were exposed to PWM (Techtum, Uppsala, Sweden) by continuous contact with this agent in culture, at a concentration of 20 μg/ml. Cells were exposed to cyclosporin-A (200 ng/ml, Sandoz, Ltd., Basel, Switzerland) similarly.

Protein-A Plaque Assay. The assay used to enumerate Ig-secreting cells was a modification of that developed by Gronowicz and co-workers (18), and was performed as previously described (5–7). Briefly, 25 μl of optimally diluted rabbit anti-human-μ-chain (1:60, vol/vol) or anti-human-γ-chain (1:100, vol/vol) sera (DAKO Immunoglobulins A/S, Copenhagen, Denmark) was mixed with 100 μl of a lymphocyte suspension, 25 μl 12% (vol/vol) protein-A SRC, 25 μl SRC-absorbed guinea pig complement (Flow Laboratories, Irvine, Scotland), and 200 μl 0.5% agar (Bacto-Agar, Difco Laboratories Inc., Detroit, MI) containing 0.05% DEAE-Dextran, and poured on 15 mm × 100-mm plastic dishes, and covered with 24 mm × 32 mm glass coverslips. This mixture was allowed to gel, and incubated at 37°C in a humidified atmosphere for 3–4 h before plaques were counted. Lymphocyte preparations were washed three to four times before being assayed.

Analysis of Experimental Results. PF estimates, for human B cells stimulatable to secrete IgM or IgG, were calculated by Poissonian analysis of the data obtained from limiting dilution experiments, as described by Lefkovits and Waldmann (9). Least-squares analysis was used to obtain the line of best fit. In preliminary experiments, 4 to 5 cell dilutions with from 6 to 10 cultures per dilution were used. In final experiments, typically from 18 to 21 cultures were analyzed at each of 5 to 6 cell dilutions (varying from 30,000 to 100 B cells per culture). Values for r (a measure of the "fit" of the line to the experimental results; a value of 1 indicates a perfect fit, one of <0.95 was taken to indicate unacceptable fit) in final experiments ranged from 0.955 to 0.999+. Values for y-intercept (a measure of where the line of best fit, as calculated by least-squares analysis, intercepts the y-axis; a value of 1 is ideal) ranged from 0.88 to 1.10. All final experiments were found to conform to single-hit kinetics, when analyzed by the χ^2 test (9). Clone size estimates were obtained using the method described by Lefkovits and Waldmann (9), using the formula:

$$c = \frac{\Sigma PFC}{n},$$

where c is the clone size, ΣPFC is the sum of the PFC seen in the responding cultures, and n is the calculated number of clones present at a given cell dilution.

Positive cultures were defined as cultures showing >4 PFC, a level greater than three standard

deviations above the mean value of *PFC* seen in cultures containing only filler cells, or no cells at all. No positive cultures were seen in groups containing only filler cells. For all calculations, B cell fractions were assumed to contain 50% B cells.

Results

Optimization of Microculture Conditions. Since it is important that culture conditions allow the growth of all, or most, inducible cells to cells secreting Ig, various culture conditions were tested to determine which were optimal for the EBV- and PWM-induced polyclonal activation of human B cells to Ig secretion. Pilot experiments were designed to determine whether filler cells were required for optimal PF estimates. Autologous T cells (nylon-wool purified), cyclosporin A-treated autologous T cells, and irradiated autologous T cells were added to the B cell fraction, and the frequency of stimulatable B cells was compared to that seen when B cells were cultured without the addition of filler cells. Activation with EBV did not require the presence of filler cells for optimal PF estimates (Table I). However, PWM activation did require the presence of filler T cells (Table I). Interestingly, the use of nonirradiated, autologous T cells as filler cells led to a marked decrease in the observed EBV-activated IgM PF (Table I); this decrease was partially corrected by culturing cells in the presence of cyclosporin-A, and completely corrected by irradiation of the T cells used as fillers (Table I). Other sources of filler cells were tested (cord blood T cells and allogeneic T cells), and found not to provide better conditions for the growth of activated B cells to Ig-secreting cells than autologous, irradiated T cells (data not shown). On the basis of these results, irradiated, autologous T cells were used as filler cells in all final limiting dilution experiments, when human B cells were activated by EBV and/or PWM.

The Precursor Frequency of EBV-Activated Human B Cells for IgM and IgG Secretion. The determination of the PF of human B cells, activated by exposure to EBV, for IgM and IgG secretion, was carried out under more stringent conditions than used for the pilot experiments described above. Approximately 20 cultures were tested per B cell dilution, with 5–6 dilutions assayed per PF determination. The results of these

TABLE I
Precursor Frequencies for IgM Secretion by Human B Cells; Results of Preliminary Experiments

	Cells placed in culture			
	B only*	B + T[‡]	B + T + cy-closporin-A[§]	B + Irradi-ated T[∥]
EBV-Driven IgM precursor frequency	0.00039[¶] ±0.00020 (1/2,660)**	0.000047 ± 0.000003 (1/21,000)	0.00014 ± 0.00005 (1/7,100)	0.00065 ± 0.00008 (1/1,500)
PWM-Driven IgM precursor frequency	<0.0000025 (<1/400,000)	—	—	0.00059 ± 0.00050 (1/1,700)

* *B* cells are non-SRC-rosette-forming cells.
[‡] *T* cells are SRC-rosette-forming cells that have been passed through nylon-wool columns twice.
[§] 200 ng/ml cyclosporin-A.
[∥] 2,000 rads.
[¶] All values are the mean ± standard error of the mean of two to three independent determinations of precursor frequency.
** The fractional equivalent of the mean decimal precursor frequency estimate shown.

experiments are shown in Table II; the data from one experiment is plotted in Fig. 1 to illustrate how the experimental data were analyzed. From 1/290 to 1/2,000 B cells were seen to be precursors for the secretion of IgM; from 1/920 to 1/3,250 were precursors for the secretion of IgG. Clone sizes, calculated by the method of Lefkovits and Waldmann (9), varied from 7.0 to 8.8 PFC per precursor for IgM, and from 13 to 22 PFC per precursor for IgG secretion, indicating that the rate of cellular division required to lead to such clone sizes falls within the expected range.

The Precursor Frequency of EBV- and PWM-activated Human B Cells for IgM Secretion. In order to find whether PWM activated a similar fraction of human B cells to Ig secretion, we compared the PF of B cells activated by EBV to that activated by PWM, for IgM secretion. The calculated PF estimates from such experiments are shown in Table III. About 20 cultures per B cell dilution and 5 dilutions per determination were used.

In the first experiment done, EBV and PWM activated comparable fractions of human B cells to IgM secretion, with IgM PF of ~1/1,000 (Table III, Experiment 1).

TABLE II

Precursor Frequencies of EBV-Activated Human B Cells for the Secretion of IgM and IgG

	B cell precursor frequency*	
	IgM	IgG
Experiment 1[‡]	0.00342	0.000307
	(1/290)[§]	(1/3,250)
Experiment 2	0.000505	0.00108
	(1/2,000)	(1/920)

* Calculated by the method of Lefkovits and Waldman (9): see Materials and Methods section.
[‡] Calculated from the data presented in Fig. 1.
[§] The fractional equivalent of the decimal precursor frequency estimate shown directly above it.
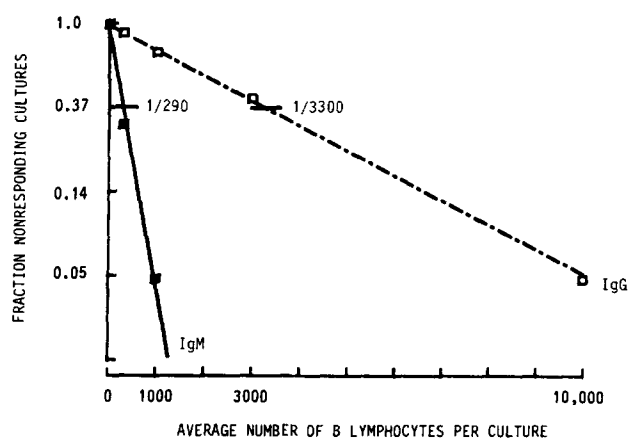

FIG. 1. Limiting dilution analysis of EBV-activated human peripheral blood B lymphocytes. Total IgM PFC (■——) and IgG PFC (□– – –) responses were determined using the indirect protein-A plaque assay technique (18). The calculated precursor frequencies are shown (1/290 for IgM, and 1/3,300 for IgG secretion). r values were 0.994 for IgM, and 0.999+ for IgG; y-intercepts were 0.88 for IgM and 0.99 for IgG.

TABLE III

The Precursor Frequencies of EBV- and PWM-Activated Human B Cells for IgM Secretion

	IgM B cell precursor frequency*		
	EBV-Activated	PWM-Activated	EBV + PWM-Activated
Experiment 1	0.000855	0.000971	—
	(1/1,200)[‡]	(1/1,000)	—
Experiment 2	0.00167	0.00735	0.010
	(1/600)	(1/140)	(1/100)
Experiment 3	0.000267	0.000310	0.000403
	(1/3,700)	(1/3,200)	(1/2,500)

* Calculated using the method of Lefkovits and Waldmann (9): see Materials and Methods section.
[‡] The fractional equivalent of the decimal precursor frequency estimate shown above it.

Since EBV and PWM seemed to activate a similar number of precursors to secrete IgM, experiments were carried out to determine if they operated on the same or different B cell subpopulation. B cells isolated from the same donor were activated with EBV, PWM, or EBV + PWM, and placed in limiting dilution cultures and assayed as described above (Table III, Experiments 2 and 3). If these two PBA were operating on the same subpopulation of B cells, the expected EBV + PWM IgM PF should equal the EBV or PWM IgM PF (whichever was greater). If they were operating on different B cell subpopulations, the combined EBV + PWM IgM PF should equal the sum of the EBV IgM PF and the PWM IgM PF. An intermediate result would indicate that the EBV and PWM stimulatable subpopulations over-lapped. The observed EBV + PWM IgM PF was seen to be approximately equal to the sum of the separate, EBV- and PWM-activated IgM PF (Table III), indicating that EBV and PWM appear to have activated different B cell subpopulations.

Discussion

In searching for the optimal culture conditions, we have found that, for the activation of human B cells by EBV, no addition of autologous T cells was needed (Table I). In fact, if taken from EBV-immune individuals, addition of such cells (nonirradiated) decreased the observed PF by nearly a factor of 10. This is compatible with Thorley-Lawson's data (19) on this point, which says that if T cells are present from the start of EBV exposure to autologous B cells, they interfere with activation. We have reason to assume that this negative effect of T cells on EBV activation of human B cells can be made quantitative, and thus used to determine the frequencies of anti-EBV specific T cells. In addition, we observed that this negative influence of live T cells is abolished if the cells are irradiated in vitro, but only partially abolished if they are exposed to cyclosporin A (Table I).

At first glance, the frequency of human B cells induced to Ig secretion by EBV may seem low, especially when compared to frequencies reported for Ig secretion by polyclonally activated murine spleen B cells (8). However, similar frequencies have been reported for PWM-induced Ig secretion by human peripheral blood B cells (10), even though these figures were obtained using a different experimental system. The observed PF and clone size estimates for IgM and IgG secretion by EBV-activated human peripheral blood B cells correlate well with the PFC response of these cells

when cultured in nonlimiting conditions (5, 6).

We assume, at this point, that these polyclonal activators can only drive a small (1/100 to 1/5,000) subpopulation of peripheral blood B cells, which are at a certain maturational stage, to Ig synthesis and secretion. As virtually every human B cell exposed to EBV in vitro is indeed infected by the virus, and accordingly expresses the viral antigens intranuclearly (5, 6), the point of decision to go towards Ig secretion must be somewhere beyond these steps in viral infection.

The similar magnitude of the EBV- and PWM-activatable cell PF (Table III) led us to question whether these PBA operate on the same B cell subpopulation, what could be called an "activatable B cell subpopulation", or whether they operate on separate, or partially overlapping subpopulations. Human B cells activatable by PWM have been shown to represent a distinct subpopulation (20, 21). To answer this question, B cells activated by EBV, PWM, and EBV together with PWM (EBV + PWM) were cultured under limiting dilution conditions (Table III). Since the EBV + PWM IgM PF was approximately equal to the sum of the separate EBV and PWM IgM PF, we have concluded that these PBA operate on separate B cell subpopulations. However, the possibility that a small fraction of PWM-activatable cells can also be activated by EBV cannot, with certainty, be excluded.

These data shed light on the repeated observations that EBV infection in vivo results in a polyclonal Ig response that has a different specificity pattern than other infectious or noninfectious conditions associated with a polyclonal Ig response.

Summary

We have developed a microculture system suitable for limiting dilution analysis of Epstein-Barr virus (EBV)- and pokeweed mitogen (PWM)-induced activation of immunoglobulin secretion by human B cells. It was found that exogenous filler cells were not required to obtain optimal EBV-induced B cell precursor frequency (PF) estimates, although filler T cells were required for optimal PWM activation. In fact, when autologous T cells were used as filler cells, a marked decrease in the EBV-induced IgM PF was noted. Treatment of the T cells with cyclosporin A partially eliminated, and irradiation of the T cells completely eliminated, this decrease.

The calculated PF of B cells activated by EBV was from 1/290 to 1/3,700 for IgM, and from 1/920 to 1/3,250 for IgG secretion. PWM activated from 1/140 to 1/3,200 B cells to IgM secretion. The results of experiments in which EBV and PWM were mixed, indicated that these two polyclonal activators operated on different B cell subpopulations. Therefore, both these agents seem to activate small, discrete subpopulations of human peripheral blood B cells to Ig secretion.

Note added in proof: After the submission of this work for publication, a report was published (Yarchoan, R., G. Tosato, R. M. Blaese, R. M. Simon, and D. L. Nelson. 1983. Limiting dilution analysis of Epstein-Barr virus-induced immunoglobulin production by human B cells. *J. Exp. Med.* **157:**1) in which similar precursor frequency figures were presented for immunoglobulin secretion by Epstein-Barr virus activated human B cells, using an assay system for secreted immunoglobulin present in cell culture supernatants, rather than a hemolytic plaque assay.

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