LIMITING DILUTION ANALYSIS OF EPSTEIN-BARR VIRUS-INDUCED IMMUNOGLOBULIN PRODUCTION BY HUMAN B CELLS

By ROBERT YARCHOAN,* GIOVANNA TOSATO, R. MICHAEL BLAESE, RICHARD M. SIMON, AND DAVID L. NELSON

From the Metabolism Branch and the Biometric Research Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Epstein-Barr virus (EBV)[1] is a herpes virus that can infect human B cells. This virus has been shown to be the causative agent in infectious mononucleosis (1) and to be associated with several other disorders including African Burkitt's lymphoma (2), nasopharyngeal carcinoma (3), and certain cases of acquired hypogammaglobulinemia (4). Many if not all human B cells have receptors for EBV that appear to be necessary for infection by the virus (5–7). B cells so infected may be induced to secrete immunoglobulin (Ig) (8) and may become transformed into long-term cell lines (9). Ig production induced by the B95-8 strain of EBV does not require the presence of either T cells or monocytes (10, 11), and this strain has found wide usage as a tool to study the functional capabilities of human B cells. All major classes of Ig are secreted by B95-8-stimulated cultures of human B cells, and the relative proportion of these classes is generally not affected by the addition of T cells (12).

Recent in vitro studies (13) have demonstrated that whereas most if not all human B cells have receptors for EBV, only a proportion of these cells can be transformed by the virus. It has in addition been inferred that only a small subset of human B cells are induced to secrete Ig by infection with EBV (8, 14). In the present experiments, we have used Poisson analysis of limiting dilution cultures to determine the precursor frequencies of human peripheral blood B cells that can be activated by EBV to secrete IgG or IgM in short-term culture. We have also demonstrated that the precursor cells are induced by EBV to secrete IgG or IgM, but not both classes of Ig. Finally, we have determined that the activation of B cells to Ig production is consistent with a "one-hit" model; that is, infection by one virion is sufficient to induce a B cell to produce Ig in short-term culture.

Materials and Methods

Preparations of Virus. Three preparations of the B95-8 strain of EBV were used in these experiments. Preparations 1 and 2 were obtained by sterile filtration of the supernatant of the B95-8 marmoset cell line through a 45-μm filter (Nalge Co., Nalgene Labware Div., Rochester, NY); these preparations had 10^4 and 10^6 umbilical cord-transforming units per ml, respectively, as determined by conventional technique (15). Preparation 3 was obtained in a similar fashion,

* To whom correspondence should be addressed at Bldg. 10, Rm. 4N113, National Institutes of Health, Bethesda, MD 20205.

[1] *Abbreviations used in this paper:* EBV, Epstein-Barr virus; ELISA, enzyme-linked immunoabsorbent assay; FCS, fetal calf serum; LPS, lipopolysaccharide; PWM, pokeweed mitogen.

except that the supernatant was first concentrated 100-fold by ultracentrifugation at 120,000 *g* for 60 min; this preparation had $>10^9$ umbilical cord transforming units per ml. Preparation 1 was used in studies of the viral requirements for B cell activation, and preparations 2 and 3 were used in B cell precursor frequency determinations.

Lymphocyte Preparation and Culture Conditions. Heparinized peripheral venous blood was obtained from healthy adult donors between the ages of 25 and 40 yr. Peripheral blood mononuclear cells were obtained by Ficoll-Hypaque centrifugation (16) and partially depleted of cytophilic Ig by incubation for 1 h at 37°C in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal calf serum (FCS) (all from Grand Island Biological Co., Grand Island, NY). B and T cell-enriched populations were prepared using columns of rabbit anti-human-$F(ab')_2$ antibody bound to Sephadex G200 as previously described (17). The B cell-enriched populations so prepared contained <2% T cells as detected by E rosetting, and the T cell-enriched populations contained <2% sIg^+ cells as determined with a fluorescein-conjugated goat anti-human Ig antisera (N. L. Cappel Laboratories, Cochranville, PA) (18). The B cell-enriched populations so prepared contained 50–65% B cells (mean 60%), as determined by the criteria of: (*a*) staining with fluorescein-conjugated anti-human Ig, and (*b*) failure to ingest latex beads (Sigma Chemical Co., St. Louis, MO). In experiments undertaken to determine the frequency of infectious EBV particles, the B cell population was further purified by E-rosette depletion as previously described (12). These B and T cell-enriched populations were further depleted of cytophilic Ig by two centrifugations through FCS (18). The cells were resuspended in media and counted with trypan blue.

B cell precursor frequencies for cells producing IgG or IgM were determined by limiting dilution analysis of multiple microcultures. Replicate cultures of 1×10^5 T cells irradiated with 1,200 rad and varying numbers of B cells were established in 96-well flat-bottomed microtiter plates (Costar, Data Packaging, Cambridge, MA) in a final volume of 0.25 ml of media consisting of RPMI 1640 supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 4 mM L-glutamine (Grand Island Biological Co.), and 10% FCS (Reheis, Armour Pharmaceutical Co., Kankakee, IL). Irradiation of the T cell-enriched population served the dual purpose of preventing Ig secretion by any contaminating B cells in this preparation and of eliminating the effect of suppressor T cells (19–21). At least 24 replicate cultures were established for each dose of B cells; in addition, at least 24 control wells were established with no added B cells. The cultures were stimulated with preparation 2 of EBV at a final dilution of 1:5 or pokeweed mitogen (PWM) (Grand Island Biological Co.) at a final dilution of 1:100. The cultures were incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2 for 14 d. The plates were then centrifuged at 450 *g* for 10 min, the supernatants were harvested, and the latter transferred to fresh microtiter plates and stored at 4°C. Assays for IgG and IgM were performed within 2 wk of harvest.

In certain experiments, macrocultures were simultaneously established in 16-mm well diam cluster tissue culture plates (Costar 3524). These macrocultures contained 10^6 irradiated T cells (1,200 rad) and 10^5 B cells in a total volume of 2.5 ml of the same media used in the microcultures. These macrocultures were likewise stimulated with the B95-8 strain of EBV (final dilution 1:5), cultured for 14 d, harvested, and the supernatants stored at 4°C until assayed.

Enzyme-linked Immunosorbent Assay (ELISA) for Total IgG and IgM. The total IgG and IgM in the culture supernatants was determined by a "sandwich" ELISA similar to that previously described (17, 18). To measure total IgG, wells of flat-bottomed ELISA plates (1-223-29; Dynatech Corp., Alexandria, VA) were coated overnight with 150 μl of a 1:600 dilution of affinity-purified goat anti-human IgG antibody in pH 9.6 carbonate buffer, washed three times with phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 (Sigma Chemical Co.) and 0.02% sodium azide (PBS-Tween) using a semiautomated washer (Miniwash; Dynatech Corp.), and filled with 100-μl aliquots of the culture supernatants diluted 1:1 in PBS-Tween with 1% FCS. Each plate also contained six serial twofold dilutions of an IgG standard ranging between 100 and 3.13 ng/ml. After a 2-h incubation at room temperature in a humidified atmosphere enriched with 5% CO_2, the wells were washed as above and incubated at room temperature with 100 μl of a 1:400 dilution of goat anti-human IgG conjugated to alkaline phosphatase (Sigma Chemical Co.) for an additional 2 h. After a final wash, the plates were

developed for 2 h with 1 mg/ml *p*-nitrophenol phosphate (Sigma Chemical Co.) in diethanolamine buffer (pH 9.8), and the optical density at 405 nm was read using a Multiskan (Flow Laboratories, Rockville, MD).

Total IgM was determined in a similar manner as previously described (17). In this case, a rabbit anti-human IgM was used to coat the substrate plates, and an alkaline-phosphatase-conjugated rabbit anti-human IgM used to detect bound IgM Ig. When this assay was used to quantitatively measure IgM produced by the macrocultures or pooled microcultures, each sample was assayed at several dilutions. Purified IgA and IgM myeloma proteins and purified IgG were used to test the specificity of the assays; the preparation of these purified Ig has been previously described (22). The lower limit of sensitivity of these assays was ~3 ng/ml of Ig.

Determination of B Cell Precursor Frequencies for IgG and IgM Production. Poisson analysis of these limiting dilution microcultures was used to determine the B cell precursor frequencies for IgG and IgM production. If a cell type being titrated in such cultures is randomly and independently distributed among a number of wells, then the number of precursors cells per well should follow the Poisson distribution (see ref. 23 for a discussion of the derivation and application of this formula:)

$$F_i = \frac{e^{-u}}{i!} \cdot u^i,$$

where F_i is the expected fraction of wells with i precursors when there are a mean of u precursors per well. If all the wells with one or more precursors are scored as positive, then F_o, the expected fraction of negative wells, is: $F_o = e^{-u}$. The mean number of precursors per well (u) is equal to the number of added B cells (N) times the precursor frequency f, and thus, the number of added B cells should be proportional to $-\ln(F_o)$. When there is a mean of one precursor per well ($u = 1$), then $F_o = e^{-1} = 0.368$. At this point, the precursor frequency (f) should be 1/N.

In these experiments, the IgG and/or IgM in the microculture supernatants were determined by ELISA. Those wells with more IgG or IgM than the arithmetic mean plus 2 SD of that found in the control wells (no B cells) were scored as positive for that isotype, and the fraction of negative wells for IgG and/or for IgM was calculated for each dose of added B cells. Most of the wells scored as "positive" in these experiments far exceeded the 2-SD cutoff. The actual number of added B cells was considered to be 60% of the added B cell-enriched populations based on the mean determination of the frequency of surface Ig-positive, latex ingestion-negative cells in these populations as described above. The B cell precursor frequency (f) for IgG and IgM production that best fits the experimental data to the formula above was estimated using the minimum χ^2 method described by Taswell (24). This calculation was performed using an iterative program written for a Wang 2200 computer (Wang Laboratories, Lowell, MA). Only those experiments where both $P > 0.05$ (goodness of fit test) and where the frequency determined by minimum chi square was within 15% of that determined by the maximum likelihood method were accepted as valid (24); most experiments far exceeded these criteria. In some experiments, the number of wells that were scored as positive for IgG only, IgM only, neither, or both was determined, and the independence of IgG and IgM production tested by χ^2 analysis with continuity correction of the resulting 2 × 2 contingency table (25).

Determination of the Frequency of Stimulating Viral Particles. Because EBV is particulate, it was hypothesized that Poisson analysis could also be used to test whether or not activation of B cells by EBV followed a one-hit model (i.e., one infecting viral particle is sufficient to stimulate Ig production) and if so, to determine the frequency of stimulating viral particles in preparations of EBV. To this end, multiple replicate microcultures of 10^5 B cells [prepared by anti-F(ab')$_2$ column plus E-rosette depletion] were stimulated with successive dilutions of preparation 1 of EBV; this number of B cells ensured that the precursors for IgM or IgG would not be limiting (see below). The same lot of FCS that the productive B95-8 marmoset line was grown in was used in these experiments to prepare the culture medium. As negative controls, multiple replicate wells were established without any added EBV. After 14 d, the cultures were harvested as above and the supernatants assayed for IgM and/or IgG by ELISA. The wells with more IgM than the mean + 2 SD of that in the control wells (no added EBV) were scored as positive, and the fraction of positive wells was calculated for each dilution of virus. Similar calculations were made for the IgG-containing wells.

Analysis of the number of EBV virions required to activate a B cell to produce either IgM or IgG was performed using a strategy modified from that of Parker (26). If p_i is the probability that i viral particles will infect a given B cell precursor in a culture containing N potential B cell precursors and a given dilution of the B95-8 supernatant, then, according to the Poisson distribution:

$$p_i = \frac{e^{-u}}{i!} \cdot u_i,$$

where u is the mean number of infecting viral particles per precursor B cell. It is assumed that u is proportional to the concentration of the B95-8 supernatant. Thus, the probability that a given cell will not be infected by any virions is: $p_0 = e^{-u}$, and the probability that none of the cells will be infected is: $P_- = p_0^N = e^{-uN}$. If we hypothesize that infection of a precursor B cell by one EBV virion is sufficient to induce it to produce Ig (one-hit model), then the probability of there being at least one B cell producing Ig in a well containing N precursors is: $P_+ = 1 - P_- = 1 - e^{-uN}$. This equation thus defines the relationship predicted by the one-hit model between the concentration of EBV-containing supernatant used to stimulate the cultures and P_+, the probability of observing a well containing Ig.

A similar approach was used to derive the predicted relationship between the dilution of virus and the probability of observing an Ig-containing well based on an alternative hypothesis, that a precursor B cell must be infected by two or more EBV virions before it is activated to secrete Ig (two-hit model). In this case, the probability of any one precursor not producing Ig is given by the sum of probabilities that it is infected by zero or one virions; thus; $p_- = p_0 + p_1 = e^{-u} + ue^{-u}$. The probability that a well with N potential B cell precursors contains at least one precursor that is infected by two virions and is activated to produce Ig is then: $P_+ = 1 - p_-^N = 1 - [e^{-uN} \cdot (1 + u)^N]$. This formula thus defines the relationship between the dilution of virus and probability of observing an Ig-containing well based on the two-hit model.

For each model, u (the mean number of infecting viral particles per B cell) can be expressed as Lx_i/N, where x_i is the dilution of the viral preparation used and L is the (unknown) number of stimulating virions in the undiluted viral preparation. The value of the parameter L for each of the two models that best fit the experimental data was estimated by the minimum χ^2 method. For the one-hit model, the program described above was used. For the two-hit model, the goodness of fit χ^2 for each value of L could be determined by substituting the above formula for P_+ into the standard formula for χ^2 yielding:

$$\chi^2 = \sum_{i=1}^{I} \frac{\{r_i - n_i [e^{-Lx_i} (1 + Lx_i/N)^N]\}^2}{n_i \cdot e^{-Lx_i} \cdot [1 + Lx_i/N]^N \cdot [1 - e^{-Lx_i} (1 + Lx_i/N)^N]},$$

where n_i is the number of wells established for each dilution of B95-8 supernatant x_i, and r_i is the number of negative wells at that concentration. L was determined by an iterative procedure to minimize this expression.

The degree of consistency of the results with the one- and two-hit models could thus be determined using the goodness of fit statistic with I–1 degrees of freedom. In addition, if either of these two models were valid, the value of L calculated for that model would give the estimated frequency of infectious virions in the undiluted supernatant.

Results

Specificity of the Assays for IgG and IgM. Studies were first undertaken to assess the specificity of the ELISA for IgG and IgM by testing the reactivity of purified human IgG and of purified human IgA and IgM myeloma proteins in these assays. As shown in Fig. 1, neither IgA nor IgM in concentrations of up to 500 ng/ml resulted in optical densities above that background when tested in the ELISA for IgG. Likewise, neither IgA nor IgG reacted in the ELISA for IgM. Thus, both assays were specific for their respective isotypes.

Determination of B Cell Precursor Frequencies. Studies were next undertaken to deter-

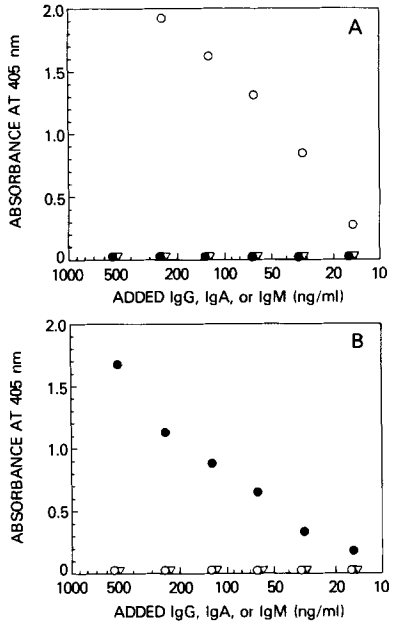

FIG. 1. Specificity of the assays used to measure IgG and IgM. (A) ELISA for IgG. Various concentrations of IgG (○), IgA (▽), and IgM (●) were tested for their reactivity in the sandwich ELISA used to measure IgG as described in the text. The optical density at 405 nm was measured after 90 min incubation with *p*-nitrophenyl phosphate. (B) ELISA for IgM. Specificity of the ELISA used to measure IgM was tested in the same manner.

TABLE I

*Comparison of the Efficiency of IgM Production in EBV-stimulated Macrocultures and Microcultures**

Culture condition	Number of B cells/culture	IgM per 10^3 added B cells
		ng
Macrocultures (10^6 T^{XR})‡	10^5	36.4 (×/÷ 1.18)§
Microcultures (10^5 T^{XR})	60	101
	120	62.3
	240	137
	480	108

* Simultaneously established macrocultures and microcultures were stimulated with EBV as described and the supernatants assayed for IgM. Macrocultures were in a total volume of 2.5 ml in 12-mm diam cluster tissue culture plates; microcultures were in a total volume of 0.25-ml in 96-well flat-bottomed microculture plates. For each dose of B cells in the microcultures, the supernatants of 24 replicate wells were pooled before being assayed.

‡ T cells irradiated with 1,200 rad.

§ Geometric mean (×/÷ SEM) of four replicate macrocultures.

mine the frequencies of peripheral blood B cells that could be activated by EBV to produce IgM or IgG. Before doing so, however, it was necessary to determine whether the efficiency of Ig production in the microculture system used in these experiments was equivalent to that in more conventional "macrocultures." As shown in Table I, the mean IgM production per added B cell in multiple microcultures, each containing

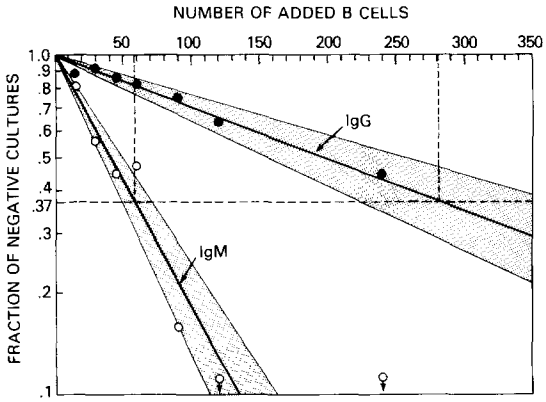

FIG. 2. Limiting dilution analysis to determine the precursor frequencies of IgM- and IgG-secreting cells in EBV-stimulated cultures. 36 replicate EBV-stimulated microcultures were established with each of various numbers of B cells and 10^5 irradiated (1,200 rad) T cells. After 14 d in culture, the supernatants were harvested and assayed for IgM and IgG. The fraction of cultures negative for IgM (○) and for IgG (●) were plotted against the number of added B cells as shown and the precursor frequencies of IgM- and IgG-secreting cells were calculated as described in the text. In this experiment, 1 in 58 added B cells produced IgM and 1 in 281 produced IgG. The stippled areas denote the 95% confidence intervals.

a limited number of B cells and 10^5 irradiated T cells, was equal to or greater than that produced in macrocultures containing 10^5 B cells and 10^6 irradiated T cells. Thus, microcultures could be used to study the activation of B cells by EBV, as the Ig produced per B cell was at least as efficient as in macrocultures.

A number of experiments were next undertaken to determine the precursor frequency of B cells that were activated by EBV to produce IgM or IgG. A representative experiment is shown in Fig. 2. As can be seen, the plot of B cell number vs. the log of the frequency of negative cultures yielded a straight line both for IgM- and for IgG-producing cells. This suggested that: (*a*) the ELISA used enabled the detection of the Ig secreted by the progeny of a single precursor B cell, and (*b*) that one cell type was limiting in these cultures. If either of these conditions were not met, the plots would yield lines that curved downward (23). The minimum χ^2 method was used to estimate the precursor frequencies; in this experiment, 1 in 58 added B cells produced IgM (1:49–1:71, 95% confidence interval) and 1 in 281 added B cells produced IgG (1:224–1:374, 95% confidence interval).

The results obtained in a number of such experiments are shown in Table II; precursor frequencies obtained in PWM-stimulated cultures are shown for comparison. The precursor frequencies for EBV-induced IgM-producing cells varied from ~1:50 to 1:220 B cells, and those for IgG-producing cells varied from ~1:150 to 1:450 B cells. It should be noted that any given precursor frequency determination represents a minimum estimate for the "actual" precursor frequency; with more efficient culture conditions, additional B cell precursors might be activated. To investigate this possibility, several different culture conditions were tested including: (*a*) an increase in the number of irradiated feeder T cells, (*b*) culturing in U-bottomed microtiter wells, and (*c*) preinfecting the B cells with EBV by incubating 2×10^6 B cells at 37° for 5 h with 5 ml of highly concentrated virus (Table III). None of these approaches yielded a significant increase in the observed precursor frequency.

TABLE II

*Determination of B Cell Precursor Frequencies for IgG and IgM Production in Cultures Stimulated with EBV or PWM**

Experiment	EBV		PWM	
	IgM	IgG	IgM	IgG
1	1:219‡	1:152	—	—
2	—	—	1:691	1:483
3	1:142	1:420	1:222	1:172
4	1:58	1:281	1:766	—
5	1:164§	—	1:3,212	—
6	—	—	1:3,093	1:3,755

* Precursor frequencies for IgM- and IgG-secreting B cells were determined in cultures stimulated with EBV or PWM as described in the text.

‡ Precursor frequencies are expressed as a fraction of the B cell population.

§ The total IgM secreted into the cultures used to determine this precursor frequency is shown in Table I.

TABLE III

*Determinations of the Precursor Frequency for EBV-induced IgM Production in a B Cell Preparation Using Different Culture Conditions**

Determination number	Number of irradiated T cells (1,200 rad)	Geometry of microtiter wells	Precursor frequency‡
1	1×10^5	Flat-bottomed	1:502 (1:356–1:852)
2	1.5×10^5	Round-bottomed	1:629 (1:427–1:1,175)
3	1.5×10^5	Round-bottomed	1:314 (1:196–1:786)

* The precursor frequency for IgM-secreting B cells was simultaneously determined using the three culture conditions described. All determinations were made using the same B cell preparation. For determinations 1 and 2, preparation 2 of EBV (10^6 virions/ml) was added directly to the cultures at a final dilution of 1:5. For determination 3, 1×10^6 B cells were incubated with 5 ml of preparation 3 of EBV ($>10^9$ virions/ml) for 5 h, appropriately diluted, and added to the cultures.

‡ Results in parentheses are the 95% confidence intervals.

By simultaneously determining the B cell precursor frequency and the IgM produced per added B cell, an estimate of the IgM produced per EBV-activated precursor B cell could be made. In Table II, experiment 5, for example, the precursor frequency of B cells activated by EBV to produce IgM was 1:164 and ~100 ng of IgM was produced per 1,000 added B cells (Table I). Thus, in this experiment, each B cell precursor activated with EBV produced ~16 ng of IgM during the 14-d culture period.

Isotype Produced by Individual B Cell Precursors. Previous studies have demonstrated that IgG-, IgA-, and IgM-secreting cells are detectable in EBV-stimulated cultures of peripheral blood mononuclear cells and that all three isotypes are produced in the absence of T cells (10–12). We next addressed the question as to whether the progeny of one precursor B cell stimulated with EBV secreted both IgM and IgG Ig or whether only a single isotype was secreted. Multiple replicate microtiter wells were established with 10^5 irradiated T cells and a limited number of B cells; the B cell number was selected based on previous precursor frequency determinations, so that each well contained approximately one-half to one B cell precursor for IgM per well. The cultures were stimulated with EBV and the supernatant of each well was assayed for both IgM and IgG. The number of wells in which both isotypes were detected was

TABLE IV

Number of Wells in which Both IgG and IgM Were Produced, and Comparison with the Number Predicted by Chance Alone

Stimulator	Experiment	Number of B cells*	Total wells	Number of wells				Predicted G^+M^+‡	χ^2	P§
				G^-M^-	G^-M^+	G^+M^-	G^+M^+			
EBV	1	60	324	130	139	26	29	28.5	0.00	>0.90
	2	60	36	15	5	11	5	4.4	0.00	>0.90
		120	36	10	4	12	10	8.6	0.44	>0.50
	5	60	288	124	110	28	26	25.5	0.00	>0.90
PWM	3	480	288	138	45	64	41	31.4	5.99	<0.02
	4	240	48	12	9	20	7	9.0	0.86	>0.30
		480	48	12	6	11	19	15.6	2.94	>0.05
	5	240	288	28	62	61	137	136.8	0.01	>0.90

* Number of B cells added to each well.

‡ Predicted number of G^+M^+ wells based on the total number of G^+ and M^+ wells.

§ Significance level for the discrepancy between the observed results and those predicted under the hypothesis that there are no precursors producing both IgM and IgG.

compared with the number predicted by chance alone if the precursors for each isotype segregated independently. As shown in Table IV, in each of three experiments the number of wells with both IgM and IgG was consistent with that predicted by chance alone ($P > 0.05$), and in most cases the number was nearly identical. Thus, the precursor cells whose progeny produced IgM were separate from those whose progeny produced IgG; in other words, a cell stimulated with EBV could be induced to secrete IgM or IgG in short-term culture, but not both.

Similar studies were undertaken in cultures stimulated with PWM (Table IV). In all but one experiment, a similar result was observed; the precursors for IgM were separate from those for IgG. In one experiment (experiment 3), however, there were slightly more wells in which IgM and IgG were detected than were predicted by chance alone ($P < 0.02$), suggesting that the progeny of a small percentage of the precursor B cells secreted both IgM and IgG. Even in this experiment, it is estimated that over 90% of the B cells stimulated by PWM produced only one isotype.

Determination of the Number of Stimulating Virions. It has previously been demonstrated that a single EBV particle is sufficient to "transform" an individual B cell into a long-term B cell line (one-hit model) (13). We addressed the question as to whether Ig production, like B cell transformation by this virus, follows a one-hit model. Because EBV is particulate, cell infection in culture with limiting numbers of virions should follow a Poisson distribution; analysis of Ig production in multiple cultures with limiting dilutions of EBV might thus enable the study of the viral requirements for B cell activation to Ig production. To this end, multiple replicate cultures were established with 10^5 B cells (surface Ig-positive and E^-) and various dilutions of preparation 1 of EBV. After 14 d, the supernatants were assayed for IgM and/or IgG and the frequency of cultures positive for IgM and/or IgG was determined for each dilution of the EBV-containing supernatant.

The first question addressed was whether the results were consistent with the hypothesis that one infecting virion was sufficient to activate a B cell to produce Ig (one-hit model) or alternatively, with the hypothesis that two or more infecting virions

were required (two-hit model). As described in Materials and Methods, the predicted mathematical relationships between the mean number of infecting virions per B cell (u) and the frequency of positive cultures can be derived based on each of these models. The curves showing these theoretical relationships are shown in Fig. 3 A. When plotted in this manner, both theoretical curves are sigmoid shaped. The two-hit curve is shifted to the left of the one-hit curve, as a greater mean number of viral particles per B cell are required to activate at least one B cell in any given fraction of cultures with the former (two-hit) model. In addition, the slope of the central linear portion is steeper with the two-hit curve than with the one-hit curve; if more than two viruses are required (three-hit, etc.), then the central part of the curve is steeper yet (curves not shown). These differences in slope can be used to visually approximate which curve most closely fits the experimental results.

Fig. 3 B shows the experimental results obtained in a typical experiment with limiting dilutions of virus in which IgM production was examined. As can be seen, the slope of the plotted experimental results is closer to that of the one-hit curve than that of the two-hit curve, suggesting that infection by one EBV virion was sufficient to induce a B cell to produce IgM. To analyze these results more rigorously, the minimum χ^2 method was used to fit the experimental results obtained to both models (Table V); in each case, the results were consistent with the curve predicted by the

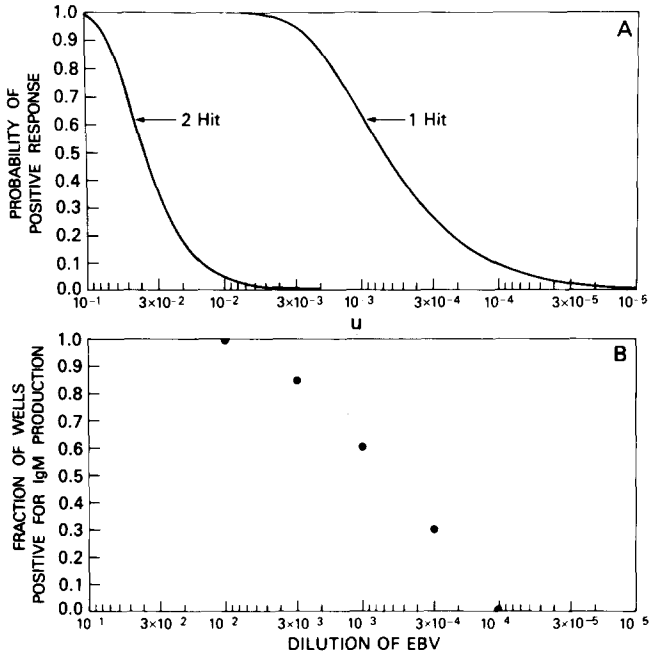

FIG. 3. The activation of B cells by EBV is consistent with a one-hit model. (A) Theoretical curves of u (the probability that any given precursor B cell is infected by a virion) vs. the probability of observing wells with secreted Ig calculated for 10^3 precursor B cells using the one-hit and two-hit models as described in the text. The two-hit curve has a steeper central portion than the one-hit curve when plotted in this manner. (B) Observed results in cultures established with limiting dilutions of EBV. 10^5 B cells in a final volume of 0.2 ml were stimulated with various dilutions of the supernatant of the B95-8 marmoset line, and the fraction of wells in which IgM was detected was calculated for each dilution of virus. As can be observed, the shape of this curve is close to that predicted by the one-hit model.

TABLE V

*Activation of B Cells by EBV to Produce Ig Is Consistent with a One-Hit but not a Two-Hit model**

Experiment	Ig class	Replicates per dilution of virus	One-hit model		Two-hit model		Activating virions/ml‡
			χ^2	P	χ^2	P	
1	IgM	20	4.04	>0.25	60.6	<0.005	7,100§ (4,880–9,300)
	IgG	20	4.63	>0.25	56.2	<0.005	11,260 (7,730–14,790)
2	IgM	60	5.87	>0.10	108.3	<0.005	6,250 (5,030–7,490)

* Multiple replicate cultures were established with 10^5 B cells and limiting dilutions of virus as described in the text. After 14 d in culture, the wells were assayed for the presence of IgM and/or IgG, and the minimum χ^2 method used to determine if these results were consistent with either the one- or two-hit model. P is the probability value as measure of the goodness of fit with the model tested.

‡ Estimate of the number of EBV virions per ml of undiluted supernatant as described in the text, based on the one-hit model. Numbers in parentheses are the 95% confidence interval.

§ The results of this experiment are also shown plotted in Fig. 3.

one-hit model but not the two-hit model.

Having shown that the activation of B cells by EBV to produce IgM or IgG is consistent with the distribution predicted by a one-hit model, it was possible to determine the concentration of stimulating EBV virions detectable in the supernatant of the B95-8 marmoset line used as a source of EBV. As shown in Table V, under these culture conditions, there were $\sim 10^4$ stimulating EBV virions detectable per ml of supernatant; similar results were obtained whether IgM or IgG activation was examined. These results were also in agreement with the determination of cord blood-transforming units in this preparation of virus (10^4 per ml).

Discussion

The results presented here demonstrate that only a small fraction of the circulating human B lymphocytes can be activated by EBV in vitro to secrete IgM or IgG. Thus, whereas almost all human B lymphocytes are believed to express receptors for EBV (6), only 0.2–1% are activated by EBV to Ig secretion.

Previous studies have attempted to determine the frequencies of B cells manifesting other features of infection by EBV. Henderson et al. (13) have estimated that up to 10% of T-depleted cord blood cells are transformed by the virus; this estimation involved a 20-fold correction for the inefficiency of their plating system. Bird et al. (27) have reported that 10–30% of B cells express EBNA, the EBV-encoded nuclear antigen, 3 d after in vitro infection with EBV. The 0.2–1% frequency of cells stimulated by EBV to secrete Ig observed here is somewhat lower than the values reported for these other two features of infection. Although this determination must be considered a lower limit for the precursor frequency, attempts to increase the observed number of precursors by altering the culture conditions or using a highly concentrated preparation of virus failed to yield significantly different results. It should be noted, however, that these precursor frequencies are for cells secreting Ig; it is possible that the frequency of cells stimulated by EBV to produce cytoplasmic Ig might be higher.

No isotype switching of secreted Ig was detectable when EBV was used as the culture stimulant; precursor cells secreted either IgM or IgG, but not both. This is unlikely to be an artifact of the assay or culture system, as most artifacts in these determinations (i.e., B cell clumping, cross-reactivity in the assay, plate-to-plate

variation in the ELISA) would lead to an overestimation of isotype switching. The failure to observe isotype switching in these short-term cultures is consistent with studies of Brown and Miller (28) who reported that almost all B cell clones obtained by EBV transformation of adult peripheral blood mononuclear cells secreted only one isotype; the few clones that appeared to secrete more than one isotype in their study could have been in reality admixtures of more than one clone. Combining their study with the present one, the Ig secreted by the progeny of any given EBV-infected peripheral blood B cell is almost always restricted to one isotype. Thus, isotype switching is not an inevitable consequence of continued B cell proliferation.

The lack of isotype switching observed here with EBV contrasts with reported studies of murine B cells. Using a similar limiting dilution technique, Anderson et al. (29) found that all of the IgG-secreting cells in lipopolysaccharide (LPS)-stimulated cultures of murine spleen cells developed by isotype switching in clones that had previously contained IgM-secreting cells; similar results have been obtained using cells obtained from a variety of murine lymphoid organs (30). Other investigators have reported the frequent detection of isotype switching in antigen-stimulated cultures of murine B cells using the splenic focus technique (31, 32). Several possible explanations can be considered for the lack of isotype switching observed here; these include (*a*) an intrinsic difference between human and murine B cells, (*b*) a difference between peripheral blood B cells and those of other lymphoid organs, or (*c*) that lack of isotype switching is a feature of stimulation by EBV. Whereas LPS is generally considered to be a T cell-independent B cell activator, previous studies have revealed that the IgG and IgA production induced by LPS is increased by the presence of T cells (33, 34). In contrast, T cells do not affect the relative proportion of the isotypes secreted in EBV-stimulated cultures of human peripheral B cells (10, 12). It was thus possible that isotype switching would only be induced when T cell-dependent or T cell-enhanced B cell activators were utilized. To investigate this hypothesis, PWM-stimulated cultures were examined. Using this T cell-dependent B cell polyclonal activator, however, no isotype switching was observed except possibly in one experiment. Even in this experiment, where more wells containing both IgG and IgM were observed than were predicted (Table IV, experiment 3), it was possible that this was an artifact due to B cell clumping or to intra-experiment variation in the culture conditions. In similar PWM-stimulated studies using 60 replicate wells per experiment, Stevens et al. (35) did not observe more wells containing multiple isotypes than predicted by chance alone. Thus, while there may be at times a low frequency of isotype switching in PWM-stimulated cultures of human peripheral blood B cells, most of the individual B cell precursors stimulated with this T cell-dependent activator secrete only a single class of Ig.

It is believed that stimulation of B cells to secrete Ig by the B95-8 strain of EBV occurs through infection of these B cells by the virus (27). With this background, we used a limiting dilutions of virus to investigate the viral requirements for B cell activation by EBV. These studies demonstrated that the activation of B cells to produce either IgM or IgG was consistent with a one-hit model and thus allowed a determination of the titer of infectious virions in the B95-8 supernatant used. It has been proposed that determinations of this sort may be affected by variations in the ability of different cells to be infected by a given virus (36). With the present experimental design, however, in which only one Ig-producing B cell precursor per

culture is required for detection, this effect should be minimal.

Transformation of umbilical cord B cells by EBV has previously (13) been shown to also follow a one-hit model using a similar approach. In addition, these results are consistent with the findings of Byrd et al. (27), who demonstrated that the number of IgM-secreting plaques in EBV-stimulated cultures was proportional to the dilution of the virus. Taken together, these studies indicate that infection of a B cell by one viral particle is sufficient to induce that cell to secrete Ig and/or to immortalize that cell. It is not clear, however, what the relationship is between these two outcomes of B cell infection by EBV and in particular, whether all cells activated by EBV to produce Ig are also transformed (37).

In conclusion, limiting dilution techniques provide a powerful tool for the study of the individual cellular events occurring in short-term cultures of lymphoid cells. Further studies using this method should allow an investigation of the precursors of B cells committed to the production of specific antibody, of other possible mediators of isotype switching, and finally of the relationship between EBV-induced transformation and Ig production.

Summary

The Epstein-Barr virus (EBV) is a herpes virus that has the capacity to infect human B cells and to induce them to secrete immunoglobulin (Ig). In the current experiments, Poisson analysis of limiting dilution cultures has been used to study the activation of human peripheral B cells by the B95-8 strain of EBV. Under the culture conditions used, 0.2–1% of peripheral blood B cells were activated by EBV to secrete IgM or IgG. In addition, when multiple replicate cultures containing limited numbers of B cells were tested for IgM and for IgG production, the precursors for IgM and IgG segregated independently; thus, individual B cell precursors matured into cells secreting IgM or IgG but not both classes of Ig. Additional experiments using limiting dilutions of EBV were undertaken to study the viral requirements for B cell activation. These studies indicated that B cell activation by EBV to produce Ig was consistent with a "one-hit" model and inconsistent with a "two-hit" model. Taken together, these results indicate that infection by one EBV virion is sufficient to induce a precursor peripheral blood B cell to secrete Ig and that only one isotype of Ig is then secreted.

The authors wish to thank Dr. Bonita M. Bundy and Ms. Donna Whitehurst for their technical assistance, Dr. Ian Magrath for preparing the concentrated virus, Dr. Jay Berzofsky for critical reading of the manuscript, and Dr. Nolan Sigal for his candid discussion of unpublished results. In addition, the helpful discussions with Dr. Warren Strober and the past guidance of Dr. P. O. Offenhartz are gratefully acknowledged.

Received for publication 13 August 1982.

References

1. Henle, G., W. Henle, and V. Diehl. 1968. Relation of Burkitt's tumor-associated-type virus to infectious mononucleosis. *Proc. Natl. Acad. Sci. U. S. A.* **59:**94.
2. Klein, G. 1972. Herpes viruses and oncogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **69:**1056.
3. Wolf, H., H. zur Hausen, and V. Becker. 1973. EB viral genomes in epithelial nasopharyngeal carcinoma cells. *Nature (Lond.).* **244:**245.

4. Provisor, A. J., J. J. Iacuone, R. R. Chilcote, R. G. Neiburger, F. G. Crussi, and R. L. Baehner. 1975. Acquired agammaglobulinemia after a life-threatening illness with clinical and laboratory features of infectious mononucleosis in three related male children. *N. Engl. J. Med.* **293:**62.
5. Jondal, M., and G. Klein. 1973. Surface markers on human B and T lymphocytes. Presence of Epstein-Barr virus receptors on B lymphocytes. *J. Exp. Med.* **138:**1365.
6. Greaves, M. F., G. Brown, and A. B. Rickinson. 1975. Epstein-Barr virus binding sites on lymphocyte subpopulations and the origin of lymphoblasts in cultured cell lines and in the blood of patients with infectious mononucleosis. *Clin. Immunol. Immunopathol.* **3:**514.
7. Volsky, D. J., G. Klein, B. Volsky, and I. M. Shapiro. 1981. Production of infectious Epstein-Barr virus in mouse lymphocytes. *Nature (Lond.).* **293:**399.
8. Rosen, A. M., M. 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.
9. Henle, W., V. Diehl, G. Kohn, H. zur Hausen, and G. Henle. 1965. Herpes type virus and chromosome marker in normal leukocytes after growth with irradiated Burkitt cells. *Science (Wash. D. C.).* **157:**1064.
10. Kirshner, H., G. Tosato, R. M. Blaese, S. Broder, and I. T. Magrath. 1979. Polyclonal immunoglobulin secretion by human B lymphocytes exposed to Epstein-Barr virus *in vitro. J. Immunol.* **122:**1310.
11. Bird, 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. Tosato, G., I. T. Magrath, I. R. Koski, N. J. Dooley, and R. M. Blaese. 1980. B cell differentiation and immunoregulatory T cell function in human cord blood lymphocytes. *J. Clin. Invest.* **66:**383.
13. Henderson, E., G. Miller, J. Robinson, and L. Heston. 1977. Efficiency of transformation of lymphocytes by Epstein-Barr virus. *Virology.* **76:**152.
14. Steel, C. M., J. Philipson, E. Arthur, S. E. Gardiner, M. S. Newton, and R. V. McIntosh. 1977. Possibility of EB virus preferentially transforming a subpopulation of human B lymphocytes. *Nature (Lond.).* **270:**729.
15. Moss, D. J., and J. H. Pope. 1972. Assay of the infectivity of Epstein-Barr virus by transformation of human leucocytes *in vitro. J. Gen. Virol.* **17:**233.
16. Böyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21**(Suppl. 97)**:**77.
17. Yarchoan, R., W. E. Biddison, H. S. Schneider, and D. L. Nelson. 1982. Human T-cell subset requirements for the production of specific anti-influenza virus antibody *in vitro. J. Clin. Immunol.* **2:**118.
18. Yarchoan, R., B. R. Murphy, W. Strober, H. S. Schneider, and D. L. Nelson. 1981. Specific anti-influenza virus antibody production *in vitro* by human peripheral blood mononuclear cells. *J. Immunol.* **127:**2588.
19. Siegal, F. P., and M. Siegal. 1977. Enhancement by irradiated T cells of human plasma cell production: dissection of helper and suppressor function *in vitro. J. Immunol.* **118:**642.
20. Tosato, G., I. T. Magrath, M. S. Koski, N. Dooley, and R. M. Blaese. 1979. Activation of suppressor T cells during Epstein-Barr virus-induced infectious mononucleosis. *N. Engl. J. Med.* **301:**1133.
21. Tosato, G., I. T. Magrath, and R. M. Blaese. 1982. T cell-mediated immunoregulation of Epstein Barr virus- (EBV) induced B lymphocyte activation in EBV-seropositive and EBV-seronegative individuals. *J. Immunol.* **128:**575.
22. Murphy, B. R., M. A. Phelan, D. L. Nelson, R. Yarchoan, E. L. Tierney, D. W. Alling, and R. M. Chanock. 1981. Hemagglutinin-specific enzyme-linked immunosorbent assay for antibodies to influenza A and B viruses. *J. Clin. Microbiol.* **13:**554.

23. Lefkovits, I. and H. Waldmann. 1979. Limiting Dilution Analysis of Cells in the Immune System. Cambridge University Press, Cambridge. 262.
24. Taswell, C. 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J. Immunol.* **126:**1614.
25. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. Sixth Edition. Iowa State University Press, Ames, Iowa. 593.
26. Parker, R. F. 1938. Statistical studies of the nature of the infectious unit of vaccine virus. *J. Exp. Med.* **67:**725.
27. Bird, A. G., S. Britton, I. Ernberg, and K. Nilsson. 1981. Characteristics of Epstein-Barr virus activation of human B lymphocytes. *J. Exp. Med.* **154:**832.
28. Brown, N. A., and G. Miller. 1982. Immunoglobulin expression by human B lymphocytes clonally transformed by Epstein-Barr virus. *J. Immunol.* **128:**24.
29. Andersson, J., A. Coutinho, and F. Melchers. 1977. Frequencies of mitogen-reactive B cells in the mouse. II. Frequencies of B cells producing antibodies which lyse sheep or horse erythrocytes, and trinitrophenylated or nitroiodophenylated sheep erythrocytes. *J. Exp. Med.* **145:**1520.
30. Benner, R., A. Coutinho, A. M. Rijnbeek, A. van Oudenaren, and H. Hooijkaas. 1981. Immunoglobulin isotype expression. II. Frequency analysis in mitogen-reactive B cells. *Eur. J. Immunol.* **11:**799.
31. Gearhart, P., N. H. Sigal, and N. Klinman. 1975. Production of antibodies of identical idiotype but diverse immunoglobulin classes by cells derived from a single stimulated B cell. *Proc. Natl. Acad. Sci. U. S. A.* **72:**1707.
32. Teale, J. M., D. Lafrenz, N. R. Klinman, and S. Strober. 1981. Immunoglobulin class commitment exhibited by B lymphocytes separated according to surface isotype. *J. Immunol.* **126:**1052.
33. Elson, C. O., R. Yarchoan, A. Graeff, and W. Strober. 1980. Lipopolysaccharide (LPS) induced murine IgA synthesis is T cell dependent. *Fed. Proc.* **39:**916.
34. Kawanishi, H., L. E. Saltzman, and W. Strober. 1982. Characteristics and regulatory function of murine Con A-induced, cloned T cells obtained from Peyer's patches and spleen: mechanisms regulating isotype-specific immunoglobulin production by Peyer's patch B cells. *J. Immunol.* **129:**475.
35. Stevens, R. H., E. Macy, and C. J. Thiele. 1981. Evidence that pokeweed-mitogen-reactive B cells are pre-committed *in vivo* to the high rate secretion of a single immunoglobulin isotype *in vitro*. *Scand. J. Immunol.* **14:**449.
36. Lauffer, M. A., and W. C. Price. 1945. Infection by viruses. *Arch. Biochem.* **8:**449.
37. Robinson, J. E., D. Smith, and J. Niederman. 1981. Plasmacytic differentiation of circulating Epstein-Barr virus-infected B lymphocytes during acute infectious mononucleosis. *J. Exp. Med.* **153:**235.