JOURNAL OF VIROLOGY, Mar. 1987, p. 774–781
0022-538X/87/030774-08$02.00/0

Vol. 61, No. 3

Synergistic Activation of Cells by Epstein-Barr Virus and B-Cell Growth Factor

LINDSEY M. HUTT-FLETCHER

Department of Comparative and Experimental Pathology, University of Florida College of Veterinary Medicine, Gainesville, Florida 32610

Received 4 June 1986/Accepted 21 November 1986

Infection with Epstein-Barr virus (EBV) is initiated by virus binding to the C3dg-C3d receptor CR2. Several workers have implicated this receptor in the control of B-cell activation by examining the effects of antibodies to CR2 and isolated C3d on B-cell proliferation and differentiation. We report here on the activating effects of irradiated EBV, which retains its capacity to bind to CR2 but loses its ability to function as a T-independent B-cell activator. EBV synergized with B-cell growth factor in the induction of uptake of tritiated thymidine by T cell-depleted leukocytes from seronegative donors but did not induce secretion of immunoglobulin. Synergism could be inhibited with an anti-viral antibody that inhibited binding of EBV to CR2. No similar synergism was found between EBV and recombinant interleukin 2, interleukin 1 alpha, or gamma interferon or with the lipid A fraction of bacterial lipopolysaccharide. EBV may thus initiate B-cell activation as it binds to CR2. Infectious virus may, under normal circumstances, induce the cell to make those growth factors necessary to support B-cell proliferation; the difficulty of transforming cells with transfected EBV DNA may in part reflect the absence of an activation event provided by intact virus as it attaches to CR2. The synergism of EBV and B-cell growth factor more clearly distinguishes the effects of B-cell growth factor from those of interleukin 1 and interleukin 2 in other models of B-cell activation. Thus, this may be a useful model for further delineation of unique effects of B-cell growth factor on B-cell function.

The tropism of Epstein-Barr virus (EBV) for human B lymphocytes (16) and for undifferentiated epithelium (38) reflects the stringent requirement of the virus for attachment to a specific cell membrane protein. This protein, CR2, binds both the C3dg fragment of complement, which is presumably the physiologic ligand, and EBV (7, 29), although probably at different sites (14). The importance of CR2 to normal B-cell function is not clear, but several groups have recently implicated it in the control of B-cell activation. Antibodies to CR2, in concert with T cells (28) or T cell-derived B-cell growth factors (BCGF) (8), induce B cell proliferation. Cross-linked human C3d, which binds to the equivalent receptor on the murine B cell, has been described as controlling the entry of B cells from G_1 into the S phase (22). We therefore thought it of interest to determine whether, by virtue of its adaptation to bind to CR2, EBV has acquired the ability to mimic some of these effects.

Any stimulatory effects that EBV might have on a B lymphocyte by triggering receptor-mediated activation are normally overshadowed by the ability of the virus to infect and transform the cell. However, after inactivation by UV irradiation the virus loses its ability to successfully infect and stimulate [^{3}H]thymidine (^{3}HTdR) incorporation or immunoglobulin secretion (2, 9). We took advantage of this to separate the effects of virus binding, which are unaffected by irradiation, from those effects of virus gene expression that are probably responsible for transformation. We also confined all our experiments to the examination of effects on T cell-depleted, B cell-enriched leukocytes from EBV-seronegative donors to avoid the possibility of measuring antigen driven proliferation of EBV-specific T cells (9, 15). To provide the T-cell help that has been reported to be necessary for B-cell activation via CR2 we added soluble growth factors. We report here that irradiated EBV, which has no effect on cell proliferation on its own, can synergize with BCGF to stimulate cell division.

MATERIALS AND METHODS

Cell preparation and culture. Heparinized peripheral blood from EBV-seronegative donors was separated by flotation on lymphocyte separation medium (Litton Bionetics, Charleston, S.C.). T cells were depleted by a double cycle of rosetting with 2-aminoethylisothiouronium bromide-treated sheep erythrocytes (31) and centrifugation over 60% Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.). These T-depleted leukocytes were incubated at 37°C in 96-well, round-bottomed tissue culture plates at concentrations of 10^5 cells per well in a total volume of 100 μl of RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (Sigma Chemical Co., St. Louis, Mo.), 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. Factors and virus added to cells were made up in similar medium, and the total culture volume was maintained at 100 μl by appropriate changes in medium volume. Virus concentrations were expressed as the concentration added as one-fifth of the final volume. Factor concentrations were expressed as the final concentration present in culture media. Synergism between BCGF and virus was tested in each experiment at three dilutions of BCGF (1/4, 1/8, 1/16) and over a range of twofold dilutions of virus (1/2–1/32). In experiments in which only one value is given for a combination of virus and BCGF, the value presented is that obtained with those concentrations of virus and BCGF that together induced the greatest amount of synergism relative to the sum of responses to virus and BCGF alone at the same dilutions. Thymidine incorporation was determined by addition of 1 μCi of ^{3}HTdR (specific activity, 5 Ci/mmol; Amersham Corp., Arlington Heights, Il.) per well for the last 8 h of culture. Cells were harvested with a multiple automated sample harvester (Bellco Glass Inc., Vineland, N.J.), and incorporation of radioactivity was measured in a liquid scintillation counter. Immunoglobulin

secreted into the supernatant medium was measured on day 12 in cultures that had been fed with an additional 100 μl of medium on day 6 of culture.

Virus production. EBV was obtained from two cell lines, the marmoset cell line MCUV5, which produces virus that transforms and activates B cells, and the P3HR1-Cl13 line (11), which is superinducible with 12-*O*-tetradecanoylphorbol-13-acetate (Sigma). Both cell lines were a gift of George Miller (Yale University, New Haven, Conn.). Radiolabeled P3HR1-Cl13 virus for binding studies was obtained by feeding cells with medium containing 100 μM hypoxanthine and 0.4 μM aminopterin, inducing them when they reached confluency (day 0) with 30 ng of 12-*O*-tetradecanoylphorbol-13-acetate per ml in the presence of 2 μCi of ^{3}HTdR (5 Ci/mmol) per ml, adding an additional 2 μCi of ^{3}HTdR (52 Ci/mmol) on day 3, and harvesting on day 7. MCUV5 virus for stimulation assays was obtained from 7-day spent culture media of cells that had not been induced with 12-*O*-tetradecanoylphorbol-13-acetate. Virus in both cases was harvested from supernatant medium that had been clarified by low-speed centrifugation. The virus was pelleted by centrifugation at 20,000 × *g* for 90 min, suspended in 1/250 of the original volume of fresh medium, and filtered through a 0.45-μm filter (Millipore Corp., Bedford, Mass.). P3HR1-Cl13 virus was used for radiolabeling because the superinducibility of P3HR1-Cl13 lines enables labeling of the virus with a higher efficiency than is possible for the MCUV5 virus, which is continuously produced at low levels from the marmoset cell line. P3HR1-Cl13 virus neither superinfects nor transforms; however, more than four times as much bindable virus is present in these preparations as in MCUV5 preparations when judged by a binding assay in which a series of twofold dilutions of virus were bound to receptor-positive cells and visualized with a monoclonal antibody to EBV and a fluorescein-conjugated anti-mouse antibody (13). All the virus used for B-cell activation studies was derived from the MCUV5 cell line. Four preparations were used which contained apparently similar amounts of virus. Each preparation induced immunoglobulin synthesis in cultures of T-depleted human lymphocytes at dilutions of up to 1/160 to 1/320. In an immunofluorescence binding assay in which 100 μl of virus at various dilutions was added to 10^6 Raji cells, each virus pool could be visualized at dilutions of 1/16 to 1/32, but not at higher dilutions. Virus was inactivated by irradiation by 1 ml of virus for 30 min in a 60-mm-diameter petri dish at 20 cm from a shortwave germicidal lamp (GTE Sylvania Inc.), after which it failed to induce immunoglobulin synthesis by T-depleted human lymphocytes.

Immunoglobulin assays. Immunoglobulin in culture supernatants was measured as previously described (12) in a double-sandwich micro-enzyme-linked immunosorbent assay (37) with appropriate concentrations of rabbit anti-human immunoglobulin, peroxidase-conjugated rabbit anti-human immunoglobulin (Cooper Biomedical Inc., Malvern, Pa.), and the substrate hydrogen peroxidase with 5-aminosalicylic acid. The colorimetric change was measured at 492 nanometers.

Factors. BCGF was obtained from Cytokine Technology International Inc., Buffalo, N.Y., and was reported to contain no residual lectin, no interleukin 2 (IL-2), and no interferon (IFN). It did not induce immunoglobulin synthesis in T-depleted leukocytes and was therefore assumed to be free of infectious EBV. Recombinant IL-2 was a gift of William Benjamin, Hoffmann-LaRoche, Nutley, N.J., recombinant interleukin 1 (IL-1) alpha was obtained from Genzyme Corp., Boston, Mass., and recombinant IFN-γ was obtained from Amgen, Thousand Oaks, Calif. Bacterial lipopolysaccharide (LPS) as the lipid A-rich fraction of phenol-extracted *Escherichia coli* O111:B4 (25) was a gift of David C. Morrison (Kansas University Medical Center, Kansas City). All factors, media, and fetal calf sera were assayed for contamination with LPS by the *Limulus* amebocyte assay (20).

Antibody production. Rabbit antibody R2, specific for EBV, was produced by intramuscular inoculation of a New Zealand White rabbit which had been prebled and tested for the absence of antibody that reacted with EBV or lymphoblastoid cell lines. The animal was inoculated at weekly intervals for 3 weeks with virus concentrated from P3HR1-Cl13 cells and emulsified in Freund complete adjuvant, rested for several months, boosted twice with virus emulsified in Freund incomplete adjuvant, and bled. The antiserum was absorbed extensively by repeated passage over a column of Raji cell protein coupled to Sepharose and by repeated incubation with pelleted Raji cells. Immunoglobulin G (IgG) was purified from the serum by affinity chromatography on protein A-Sepharose (Sigma), and the protein concentration in the final preparation was determined by the method of Lowry et al. (21). The purified IgG did not react with Raji cells in an immunofluoresce assay; it did, however, react with both MCUV5 and P3HR1-Cl13 cells and with virus produced from these cell lines, and it immunoprecipitated virus proteins (data not shown). IgG purified by protein A affinity chromatography from an unimmunized rabbit that did not have anti-virus or anti-cell antibodies was used as a control reagent (nonreactive antibody).

Virus binding. The ability of antibody to inhibit binding of radiolabeled virus to receptor-positive cells was measured by preincubating virus for 1 h at room temperature with R2 antibody, nonreactive antibody, or RPMI medium containing 10% heat-inactivated fetal calf serum and incubating samples of the mixture for 1 h at 37°C with cells that had been briefly fixed in 0.1% paraformaldehyde (35). The cells were then washed five times, and the trichloroacetic acid-insoluble counts that remained associated with them were counted and compared with the total acid-insoluble counts added to the cells from the preincubation mixtures. The cells used were receptor positive Raji cells (32) and Daudi cells (19), which currently in our laboratory do not express functional CR2.

Autoradiography. Autoradiography was done as previously described (15). Briefly, leukocytes were pulsed for 1 h on day 5 with 1 μCi of ^{3}HTdR (60 Ci/mmol) per well, washed five times, and rosetted overnight. Rosetted cells were air dried on glass slides, fixed for 3 min in methanol, dipped in emulsion (Kodak Nuclear Track Emulsion NTB-2; Eastman Kodak Co., Rochester, N.Y.) at 43°C, air dried, and developed after 48 h with D19 developer (Eastman Kodak). Developed slides were stained with Diff-Quick stain (American Scientific Products, Inc., McGaw Park, Ill.) and examined by light microscopy. This procedure preserved rosettes made with sheep erythrocytes.

RESULTS

Effects of irradiated virus and BCGF on thymidine uptake by T-depleted leukocytes from seronegative donors. An initial experiment in which thymidine uptake was measured in cultures terminated between days 3 and 7 confirmed that irradiated virus induced cells to incorporate no more thymidine than they do in culture medium alone (Fig. 1). BCGF did induce a significant amount of thymidine incorporation,

which presumably represented, at least in part, the effect of factors on cells preactivated in vivo or during isolation (26). This response to BCGF was, however, augmented when virus was added simultaneously. The augmented response peaked between 5 and 7 days in culture; since it was not possible to measure incorporation on several days without limiting other variables, subsequent experiments of this type were terminated at 5 days. The synergism of BCGF and virus was predictably dependent on the concentrations of both virus and BCGF (Fig. 2), although the optimal concentration of both varied from donor to donor, experiment to experiment, and batch to batch. Optimal responses obtained in 10 experiments with seronegative donors varied considerably in magnitude (Fig. 3), but in all cases the effect of virus and BCGF together were greater than the sum of the responses to virus and BCGF alone.

Effects of EBV and BCGF on the secretion of immunoglobulin. Infection of B lymphocytes by live EBV induces not only transformation but also secretion of immunoglobulin. We therefore examined whether BCGF and irradiated virus would acquire the ability not only to induce thymidine incorporation, but also to induce immunoglobulin secretion. Parallel cultures were set up in which thymidine incorporation was measured after 5 days of culture, and the amount of immunoglobulin in supernatants was measured after 12 days. In experiments in which there was a modest augmentation by EBV of the thymidine incorporation induced by BCGF, no similar augmentation of immunoglobulin secretion was seen (Table 1). BCGF had little effect on the ability of live virus to induce thymidine incorporation and no consistent effect on the secretion of immunoglobulin.

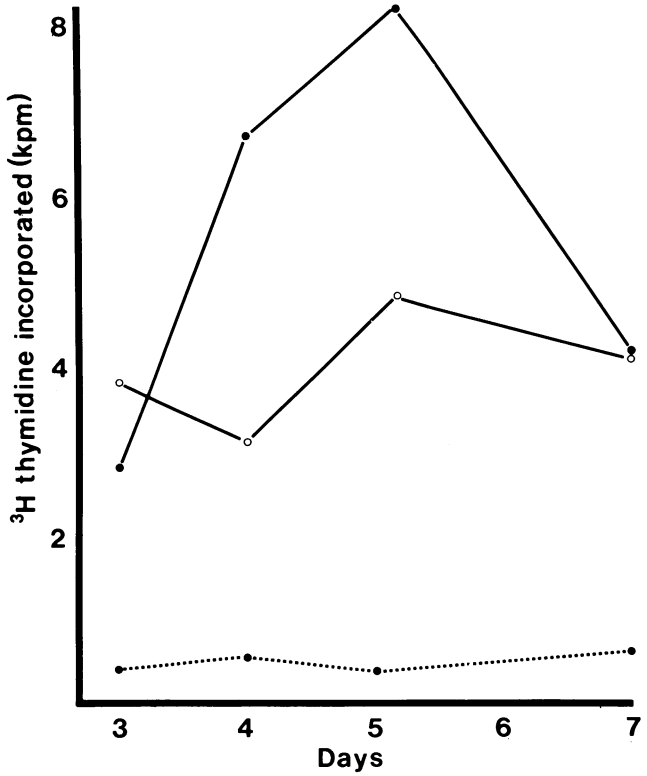

FIG. 1. Effect of irradiated EBV at a 1/32 dilution and BCGF at a 1/10 dilution on the uptake of ^{3}HTdR by T-depleted leukocytes from EBV-seronegative donors between 3 and 7 days in culture. Symbols: (●----●) virus alone, (○——○) BCGF alone, (●——●) virus and BCGF.

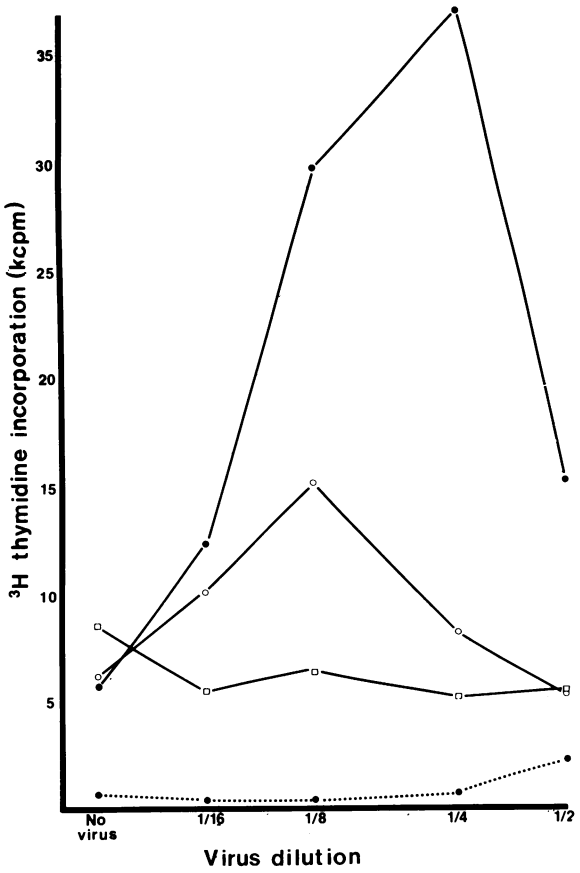

FIG. 2. Effect of different concentrations of BCGF and virus on the uptake of ^{3}HTdR by T-depleted leukocytes from EBV-seronegative donors after 5 days in culture. Symbols: (●----●) no BCGF, (●——●) BCGF at 1/4, (○——○) BCGF at 1/8, (□——□) BCGF at 1/16.

Effect of EBV and other cytokines on thymidine uptake by T-depleted leukocytes from seronegative donors. Human BCGF is not yet available as a single protein isolated to homogeneity. We therefore examined the effects of three other cytokines, IL-2, IL-1, and IFN-γ, which might be expected to be contaminants of product isolated from mitogen-stimulated human leukocytes and which are available as recombinant molecules. Recombinant IL-2 was used at final concentrations of 4 and 2 U since these amounts produced a three- to fivefold synergism (data not shown) in thymidine incorporation by cells exposed to 15 μg of anti-IgM immobilized on polyacrylamide beads (Bio-Rad Laboratories, Rockville Center, N.Y.) per ml. In experiments in which the synergism of EBV and BCGF was considerable, recombinant IL-2 had little or no effect on the response of cells to EBV (Table 2). The effects of recombinant IFN-γ were measured over a range of concentrations of 3 to 1,000 U/ml. Representative data are shown only for concentrations of 10 to 100 U/ml (Table 3), but no synergism was seen at any of the concentrations used. The effects of recombinant IL-1 alpha were measured over a range of concentrations of 3 to 250 U/ml; the values at each concentration were similar to those shown for concentrations of 10 to 100 U/ml (Table 3). No synergism was observed.

Each factor used in these experiments was checked for

TABLE 1. Effects of BCGF on ^{3}HTdR incorporation and immunoglobulin secretion by leukocytes in the presence or absence of live or UV-irradiated EBV[a]

Expt	BCGF	^{3}HTdR incorporation (cpm)			Immunoglobulin production (ng/ml)		
		Live virus	Irradiated virus	RPMI	Live virus	Irradiated virus	RPMI
1	−	5,695	266	100	8,600	290	470
	+	5,747	4,267	997	11,629	600	580
2	−	6,001	614	124	17,345	285	153
	+	5,949	5,842	3,401	8,627	443	719
3	−	4,272	781	183	10,734	100	136
	+	3,031	3,295	1,444	5,684	504	541

[a] Live and irradiated virus were used at the same dilutions.

contamination with LPS, which has been reported to induce ^{3}HTdR uptake in human B cells held in culture for a minimum of 7 days with concentrations of 1 to 400 μg of LPS per ml (24). The recombinant IFN, IL-1, and IL-2 and all sera and media used contained less than 0.05 ng of LPS per ml (the limit of detection of the assay used). One of the two lots of BCGF, that used in Fig. 1 through 3 and Tables 1 and 2 would have contributed less than 0.25 ng of LPS per ml to cultures, and that used in the remaining experiments would have contributed up to 0.25 μg of LPS per ml at the highest concentration used. Isolated LPS in these concentration ranges did not synergize with virus in the induction of thymidine incorporation by leukocytes (Table 4), and a similar absence of synergism was seen when concentrations were increased up to 1 μg or reduced to 0.063 ng in twofold dilutions (data not shown). However, small amounts of LPS in the BCGF may have contributed to the thymidine uptake induced by BCGF in the absence of virus.

Effect of EBV-specific antibody on the synergism between EBV and BCGF. The EBV used in this work was concentrated from the supernatant media of B-lymphoblastoid lines. Although we avoided the use of phorbol esters in preparation of virus from MCUV5 cells, it was possible that other potentially stimulatory materials had been concentrated together with the virus and were responsible for effects that we were attributing to the virus. To examine this possibility we required an antibody capable of blocking attachment of EBV to the EBV receptor, which, if EBV binding to CR2 was responsible for the synergism seen with BCGF, should also block increased thymidine uptake. Since none of our monoclonal antibodies to EBV completely blocked virus attachment, we prepared and purified a rabbit anti-EBV antibody.

Relatively high concentrations of this antibody were capable of blocking the attachment of radiolabeled EBV to the receptor-positive Raji cell line, whereas similar concentrations of rabbit immunoglobulin purified from normal rabbit serum did not (Table 5). The rabbit anti-EBV antibody that blocked EBV binding, but not the normal rabbit immunoglobulin, which did not, reduced the synergism between EBV and BCGF (Table 6).

Further indication that virus was responsible for the synergism with BCGF was obtained by testing, in parallel, the synergism of BCGF with "mock" virus prepared by passing the virus stock two times through a sterile 0.2-μm filter. In each of three experiments in which a modest degree of synergism was seen with virus and BCGF, the synergism was eliminated by filtration of the virus stock (Table 7).

Autoradiography of cells responding to EBV and BCGF. Although leukocyte cultures depleted by two cycles of rosetting with sheep erythrocytes contained on the average fewer than 2% rosetting cells and although all donors lacked detectable EBV antibodies, we wished to completely eliminate the possibility that the cells responding to EBV and BCGF were not residual antigen-reactive T cells. Each culture contained 1×10^5 leukocytes and thus potentially as many as 1×10^3 T cells. To test this possibility, cultures in which synergism was observed between virus and BCGF were labeled with ^{3}HTdR, rosetted, and examined by autoradiography. In an experiment in which ^{3}HTdR incorporated into cells incubated with virus alone, BCGF alone, or BCGF plus virus was 935, 5,112, and 10,233 cpm, respectively, cultures incubated with BCGF and virus contained 2% cells that rosetted with sheep erythrocytes and 21% cells that incorporated ^{3}HTdR. None of the cells that incorporated ^{3}HTdR rosetted with sheep erythrocytes.

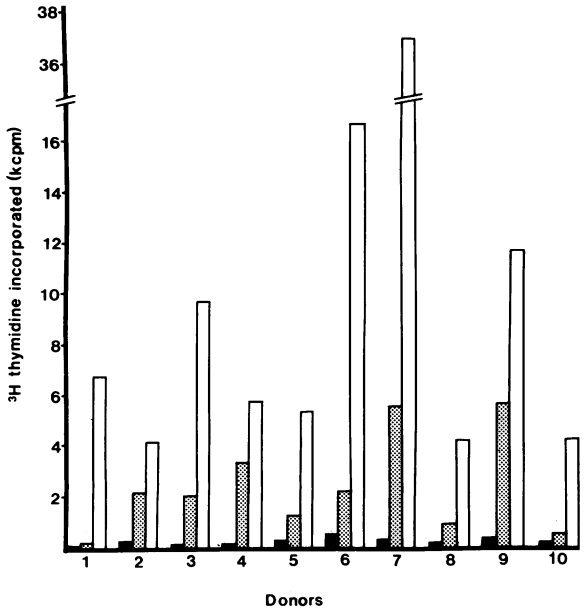

FIG. 3. Optimal uptake of ^{3}HTdR by T-depleted leukocytes from EBV-seronegative donors in response to BCGF and virus in 10 sample experiments. Symbols: (■) response to virus alone, (▩) response to BCGF alone, (▭) response to virus and BCGF.

DISCUSSION

Espstein-Barr virus infects, activates, and transforms human B lymphocytes and induces them to secrete immunoglobulin. The concept of B cell triggering and activation as a consequence of receptor-ligand interactions at the cell sur-

TABLE 2. Synergistic effects of virus and BCGF or virus and IL-2 on incorporation of ^{3}HTdR by leukocytes

Expt	Virus dilution	^{3}HTdR incorporation (cpm) by virus incubated with:					
		No addition	BCGF			IL-2	
			1/4	1/8	1/16	2 U	4 U
1	None	819	5,618	6,218	8,543	3,229	2,875
	1/4	652	37,066	9,829	5,006	4,024	1,195
	1/8	369	29,793	15,126	6,425	1,152	2,546
	1/16	423	12,483	9,100	5,429	1,749	3,351
2	None	564	2,355	1,359	1,778	674	1,365
	1/6	573	16,709	2,509	2,069	939	2,374
	1/12	977	6,291	1,358	1,386	659	1,349

face is well established (17, 18). However, it has been shown by several workers that irradiated virus, despite its apparently undiminished capacity to bind to its receptor, fails to mediate any of these events (2, 9, 34). It has thus generally been assumed that B-cell activation by EBV is related to the expression of virus genes (36).

Several recent observations challenge this assumption. All relate to the appreciation that EBV binds to a membrane protein of molecular weight 145,000, which also functions as the C3dg-C3d complement receptor (CR2) and which has been implicated in B-cell activation. Several groups have studied the effects of antibodies (8, 28; R. T. Perri, B. Wilson, and N. E. Kay, Blood 64[Suppl. 1]:79a, 1984) and C3d on B-cell activation (6, 22); however, this is a direct description of the consequences of EBV-receptor interactions.

Our data are in agreement with the concept that CR2 is involved in the regulation of B-cell activity. EBV is known to induce proliferation of mononuclear cells by two routes, by infection of the B cell or by stimulation of antigen-specific T cells. In our experiments the use of seronegative donors, the depletion of T cells from activated cultures, and the confirmation that cells taking up ^{3}HTdR do not rosette with sheep erythrocytes indicate that we are not measuring T-cell proliferation. The failure of irradiated virus alone to affect ^{3}HTdR uptake indicates that we are not measuring the T cell-independent polyclonal activation associated with EBV infection and transformation. There are both similarities and differences between the events that we describe here and those previously reported by others. All reports agree that B-cell activation by agents that bind to CR2 is a T-dependent phenomenon and describe a response that peaks slightly later than B-cell activation driven by anti-IgM or mitogen. The reports of Frade and colleagues (8) are similar to ours in that they found synergism between polyclonal antibody to C3d and activated T-cell supernatants, although no attempts were made by these workers to identify the factors in the supernatants that were responsible for the synergism that they measured. The work of Nemerow and Cooper (28) indicated that antibodies to CR2 have a more profound effect on B-cell activation than we find with irradiated EBV, in that monoclonal antibodies induced not only B-cell proliferation but also differentiation into cells capable of secreting immunoglobulins. Also, the mere presence of T cells, without stimulation by mitogens such as those used for the production of lymphokines, was sufficient to accomplish these effects. The fact that EBV and BCGF did not activate B cells to secrete immunoglobulin may mean that factors in addition to BCGF are required to complete the differentiation pathway. Several workers have suggested that discrete factors are sequentially necessary for proliferation and differentiation of B cells to follow membrane triggering signals, and synergism between BCGF and anti-IgM is reported to induce proliferation without differentiation to immunoglobulin synthesis (17, 18). However, recent cloning and analysis of a BCGF-like factor in the mouse indicated that, rather than representing the response to several different factors, the activation and differentiation of B cells represent the different responses of a changing cell to the same factor (30). The various outcomes of and requirements for B-cell activation by antibody and EBV may then reflect differences in the initial signal(s) provided by each. The work of Nemerow and Cooper (28) indicated that CR2 contains multiple functional epitopes, only some of which mediate B-cell activation. Of four monoclonal antibodies reacting with the receptor, two induced proliferation and differentiation, one induced some proliferation but no differentiation, and one induced neither. The antibody that most efficiently blocked both C3d and

TABLE 3. Synergistic effects of virus and BCGF or virus and IL-1 or IFN-γ on incorporation of ^{3}HTdR by leukocytes

Expt	Virus dilution	^{3}HTdR incorporation (cpm) by virus incubated with:						
		No addition	BCGF	IL-1			IFN-γ	
				10 U	30 U	100 U	10 U	30 U	100 U
1	None	631	3,577	603	936	502	771	495	869
	1/4	878	11,198	542	610	568	498	672	698
	1/8	1,020	7,907	460	437	872	469	597	751
	1/16	519	7,692	1,006	410	960	682	545	558
2	None	495	5,612	651	973	685	468	512	336
	1/4	309	11,584	389	722	694	270	310	413
	1/8	607	9,761	274	1,114	1,328	374	330	432
	1/16	518	7,419	555	1,615	715	396	330	457
	1/32	939	7,171	1,294	845	1,190	389	314	358

TABLE 4. Synergistic effects of virus and BCGF or virus and LPS on incorporation of ^{3}HTdR by leukocytes

Expt	Virus dilution	^{3}HTdR incorporation (cpm) by virus incubated with:				
		No addition	BCGF[a]	LPS		
				0.250 μg	0.125 μg	0.250 ng
1	None	796	6,618	6,700	6,682	2,477
	1/4	364	20,149	4,535	3,990	1,934
	1/8	529	27,784	5,424	5,023	2,659
	1/16	522	20,223	7,730	7,537	2,658
2	None	849	3,935	1,262	1,146	912
	1/4	1,415	6,477	1,703	1,533	888
	1/8	1,331	5,350	1,894	1,277	770
	1/16	1,406	5,348	1,257	964	1,644

[a] BCGF contributed 0.125 μg of LPS in these experiments.

EBV binding to CR2 was one of the two that induced both proliferation and differentiation and an antibody that, in conjunction with a second anti-immunoglobulin inhibited only C3d binding, induced only proliferation. However, EBV is a large virus which contains multiple copies of the protein(s) presumed to bind to the receptor and might perhaps be expected to have effects different from those of a molecule such as antibody, which has only two receptor binding sites and, relative to virus, an unknown affinity.

The factor(s) responsible for synergism with EBV can as yet only be operationally defined as BCGF, since a human BCGF distinct from other factors has not yet been purified to homogeneity or produced by recombinant DNA technology. We were, however, able to exclude participation of several likely contaminants of an activity purified from supernatants of mitogen-stimulated mononuclear cells which have been reported to support activation of B cells. Most interesting was the absence of synergism with recombinant IL-2, which others have reported (27) and we have confirmed to synergize with anti-IgM. Likewise, recombinant IFN-γ which has also been shown to have BCGF-like activity (33), and IL-1 alpha did not synergize with EBV over wide ranges of concentrations.

Several workers (3, 10) have recently demonstrated that B-cell lines established by transformation with EBV produce BCGF-like activity, and it has been suggested that virally transformed cells use this activity to sustain their own growth. Presumably, the EBV-producing marmoset cells from which the virus was obtained also secrete similar factors. However, it is unlikely that such factors were responsible for the effects that we are attributing to virus. First, virus was pelleted out of supernatant medium, suspended in fresh growth medium, and diluted before use. Thus, concentrations of the original supernatant media would have been several orders of magnitude less than those used to demonstrate synergism with anti-IgM. Second, when the virus was passed through a 0.2-μm filter it no longer synergized with BCGF, indicating that synergistic activation was associated with an insoluble particle. Third, antibody specific for EBV could reproducibly reduce synergism between EBV and BCGF, suggesting that virus was involved in the activation event. Synergism was not completely abrogated, but this may reflect the difficulty of completely inhibiting EBV binding with an anti-viral antibody. Monoclonal antibodies which inhibited EBV binding were not available to us from commercial sources or other investigators, and none from our own fairly extensive collection completely inhibited binding in either the presence or absence of second antibodies. The rabbit antibody we prepared inhibited binding only at fairly high concentrations of 80 μg/ml (although it neutralized the ability of virus to induce immunoglobulin synthesis at antibody concentrations of 0.3 μg/ml; data not shown), and even though the relative concentrations of antibody used to measure binding inhibition and to block synergism with BCGF were similar it seems possible that, over 5 days in culture, virus-antibody dissociation may have occurred. Final definitive proof that EBV binding is indeed responsible for B-cell triggering will require development and analysis of the effects of panels of monoclonal antibodies that variously inhibit viral binding, viral penetration, and subsequent stages of infectivity.

The susceptibility of activated B cells to infection with EBV is controversial, since some studies have indicated that

TABLE 5. Effect of antibody on the ability of radiolabeled virus to bind to EBV receptor-positive Raji cells[a]

Rabbit antibody (mg/ml)	cpm bound/cpm added	% Binding
R2 (0.16)	530/7,055	7.5
R2 (0.08)	1,622/6,790	23.8
R2 (0.04)	1,738/6,045	28.8
R2 (0.02)	1,720/5,562	30.9
R2 (0.01)	1,757/5,267	33.3
NRA[b] (0.16)	1,532/5,517	27.7
None	1,670/5,292	31.5

[a] The counts per minute bound to a receptor-negative cell control were 345/4,987 or 6.9%

[b] NRA, Nonreactive antibody.

TABLE 6. Effect of antibody on the ability of virus to synergize with BCGF

Rabbit antibody (mg/ml)[a]	Virus[b]	^{3}HTdR incorporation (cpm)			
		Expt 1		Expt 2	
		No BCGF	BCGF	No BCGF	BCGF
None	−	130	374	87	238
	+	151	5,094	82	4,264
NRA[c]	−	273	609	469	60
	+	372	5,665	400	4,316
R2	−	141	488	991	71
	+	1,136	2,289	776	2,566

[a] The concentration of antibody was 0.16 mg/ml during preincubation with virus; after addition to cells the antibody concentration was reduced fivefold.

[b] The concentrations of virus and BCGF are those that in each experiment produced maximal synergism in the absence of antibody.

[c] NRA, Nonreactive antibody.

TABLE 7. Effect of filtration on the ability of virus to synergize with BCGF

Addition	^{3}HTdR incorporation (cpm)		
	Expt 1	Expt 2	Expt 3
RPMI	590	527	333
Virus	632	549	387
BCGF	5,672	6,240	9,663
Virus plus BCGF	8,974	8,406	14,083
Filtered virus[a] plus BCGF	5,355	6,621	9,933

[a] Filtered virus was passed twice through a 0.2-μm filter and used at the same dilution as the unfiltered material.

only small resting B cells can be infected (1, 5) and others have shown that the susceptible population is heterogeneous with respect to cell size and cell cycle (4). However, even those workers whose data indicate that measurably preactivated cells are resistant to infection have suggested that the virus may infect only the population that is early in the process of activation (1). We might therefore speculate that EBV has adapted to bind exclusively to CR2, because a first signal for B-cell activation can be delivered via this molecule. In our artificial experimental system, in which gene expression by EBV has been inhibited, T cell-derived BCGF must be added to drive the cell; under normal conditions the EBV infected cell (as a result of virus gene expression?) has been shown to produce its own BCGF (3, 10). The necessity for activation to be initiated at the cell surface as EBV attaches to its target cell might be one of the reasons why it has, so far, been difficult to transform B cells with transfected DNA (23).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI20662 from the National Institutes of Health.

Thanks are due to Ann McNicol for excellent technical assistance, to Howard Johnson for many helpful discussions, and to Jennifer Johns for help with the manuscript.

LITERATURE CITED

1. **Aman, P., B. Ehlin-Henriksson, and G. Klein.** 1984. Epstein-Barr virus susceptibility of normal human B lymphocyte populations. J. Exp. Med. **159:**208–220.
2. **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:**41–67.
3. **Blazar, B. A., L. M. Sutton, and M. Strome.** 1983. Self-stimulating growth factor production by B-cell lines derived from Burkitt's lymphomas and other lines transformed in vitro by Epstein-Barr virus. Cancer Res. **43:**4562–4568.
4. **Chan, M. A., L. D. Stein, H.-M. Dosch, and N. H. Sigal.** 1986. Heterogeneity of EBV-transformable human B lymphocyte populations. J. Immunol. **136:**106–112.
5. **Einhorn, L., and E. Klein.** 1981. EBV infection of mitogen-stimulated human B lymphocytes. Int. J. Cancer **27:**181–183.
6. **Erdei, A., F. Melchers, T. Schulz, and M. Dierich.** 1985. The action of human C3 insoluble or cross-linked form with resting and activated B lymphocytes. Eur. J. Immunol. **15:**184–188.
7. **Fingeroth, J. D., J. J. Weis, T. F. Tedder, J. L. Strominger, P. A. Biro, and D. T. Fearon.** 1984. Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. Proc. Natl. Acad. Sci. USA **81:**4510–4514.
8. **Frade, R., M. C. Crevon, M. Barel, A. Vasquez, L. Krikorian, C. Charriaut, and P. Galanaud.** 1985. Enhancement of human B cell proliferation by antibody to the C3d receptor, the gp140 molecule. Eur. J. Immunol. **15:**73–76.
9. **Gerber, P., and S. J. Lucas.** 1972. Lymphocyte stimulation by Epstein-Barr virus. Cell. Immunol. **5:**318–324.
10. **Gordon, J., S. C. Ley, M. D. Melamed, L. S. English, and N. C. Hughes-Jones.** 1984. Immortalized B lymphocytes produce B-cell growth factor. Nature (London) **310:**145–147.
11. **Heston, L., M. Rabson, N. Brown, and G. Miller.** 1982. New Epstein-Barr variants from cellular subclones of P3J-HR-1 Burkitt lymphoma. Nature (London) **295:**160–163.
12. **Hutt-Fletcher, L. M., N. Balachandran, and M. H. Elkins.** 1983. B cell activation by cytomegalovirus. J. Exp. Med. **158:**2171–2176.
13. **Hutt-Fletcher, L. M., N. Balachandran, and P. A. LeBlanc.** 1986. Modification of Epstein-Barr virus replication by tunicamycin. J. Virol. **57:**117–123.
14. **Hutt-Fletcher, L. M., E. Fowler, J. D. Lambris, R. J. Feighny, J. G. Simmons, and G. P. Ross.** 1983. Studies of the Epstein-Barr virus receptor found on Raji cells. II. A comparison of lymphocyte binding sites for Epstein-Barr virus and C3d. J. Immunol. **130:**1309–1312.
15. **Hutt-Fletcher, L. M., and C. J. Gilbert.** 1981. Cytotoxic cells induced in vitro by Epstein-Barr virus antigens. Cell. Immunol. **63:**91–105.
16. **Jondal, M., and G. Klein.** 1973. Surface markers on human B and T lymphocytes. J. Exp. Med. **138:**1365–1378.
17. **Kehrl, J. H., A. Muraguchi, J. L. Butler, R. J. M. Falkoff, and A. S. Fauci.** 1984. Human B cell activation, proliferation and differentiation. Immunol. Rev. **78:**75-96.
18. **Kishimoto, T., K. Yoshizaki, M. Kimoto, M. Okada, T. Kuritani, H. Kikutani, K. Shimizu, T. Nakagawa, N. Nakagawa, Y. Miki, H. Kishi, K. Fukunaga, T. Yoshikubo, and T. Taga.** 1984. B cell growth and differentiation factors and mechanism of B cell activation. Immunol. Rev. **78:**97–118.
19. **Klein, E., G. Klein, S. Nadkarni, H. Wigzell, and P. Clifford.** 1968. Surface IgM-kappa specificity on a Burkitt lymphoma cell *in vivo* and in derived culture lines. Cancer Res. **28:**1300–1310.
20. **Levine, J., P. A. Tomasulo, and R. S. Oser.** 1970. Detection of endotoxin in human blood and demonstration of an inhibitor. J. Lab. Clin. Med. **75:**903–911.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
22. **Melchers, F., A. Erdei, T. Schulz, and M. P. Dierich.** 1985. Growth control of activated, synchronized murine B cells by the C3d fragment of human complement. Nature (London) **317:**264–267.
23. **Miller, G., E. Grogan, L. Heston, J. Robinson, and D. Smith.** 1981. Epstein-Barr viral DNA: infectivity for human placental cells. Science **212:**452–655.
24. **Miller, R. A., S. Gartner, and H. S. Kaplan.** 1978. Stimulation of mitogenic responses in human peripheral blood lymphocytes by lipopolysaccharide: serum and T helper cell requirements. J. Immunol. **121:**2160–2164.
25. **Morrison, D. C., and L. Leive.** 1975. Fractions of lipopolysaccharide from Escherichia coli O111:B4 prepared by two extraction procedures. J. Biol. Chem. **250:**2911–2919.
26. **Muraguchi, A., J. L. Butler, J. H. Kehrl, and A. S. Fauci.** 1983. Differential sensitivity of human B cell subsets to activation signals delivered by anti-antibody and proliferative signals delivered by a monoclonal B cell growth factor. J. Exp. Med. **157:**530–546.
27. **Nakagawa, T., T. Hirano, N. Nakagawa, K. Yoshizaki, and T. Kishimoto.** 1985. Effect of recombinant IL-2 and γ-IFN on proliferation and differentiation of human B cells. J. Immunol. **134:**959–966.
28. **Nemerow, G. R., and N. R. Cooper.** 1985. Binding of monoclonal antibody to the Epstein-Barr virus (EBV)/CR2 receptor induces activation and differentiation of human B lymphocytes. J. Immunol. **135:**3068–3073.
29. **Nemerow, G. R., R. Wolfert, M. E. McNaughton, and N. R. Cooper.** 1985. Identification and characterization of the Epstein-Barr virus receptor on human B lymphocytes and its relationship to the C3d complement receptor (CR2). J. Virol. **55:**347–357.
30. **Noma, Y., P. Sideras, T. Naito, S. Bergstedt-Linquist, C. Azuma, E. Severinson, T. Tanabe, T. Kinashi, F. Matsuda, Y. Yaoita,**

and T. Honjo. 1986. Cloning of cDNA encoding the murine IgG1 induction factor by a novel strategy using SP6 promoter. Nature (London) **319**:640–646.

31. **Pellegrino, M. A., S. Ferrone, M. P. Dietrich, and R. A. Reisfeld.** 1975. Enhancement of sheep red blood cell human lymphocyte rosette formation by the sulphydryl compound 2-amino-ethylisothiouronium. Clin. Immunol. Immunopathol. **3**:324–333.
32. **Pulvertaft, R. J. V.** 1964. Cytology of Burkitt's tumor (African lymphoma). Lancet **i**:238–240.
33. **Romagnani, S., M. G. Guidizi, R. Biagiott, F. Almerigogna, C. Mingari, E. Maggi, C.-M. Liang, and L. Moretta.** 1986. B cell growth factor activity of interferon-γ: recombinant human interferon-γ promotes proliferation of anti-μ-activated human B lymphocytes. J. Immunol. **136**:3513–3516.
34. **Schooley, R. T., B. F. Hayes, C. R. Payling-Wright, J. E. Grouse, R. Dolin, and A. S. Fauci.** 1980. Mechanism of Epstein-Barr virus-induced human B-lymphocytes activation. Cell. Immunol. **56**:518–525.
35. **Simmons, J. G., L. M. Hutt-Fletcher, E. Fowler, and R. J. Feighny.** 1983. Studies of the Epstein-Barr virus receptor found on Raji cells. J. Immunol. **130**:1303–1308.
36. **Tosato, G., and R. M. Blaese.** 1985. Epstein-Barr virus infection and immunoregulation in man. Adv. Immunol. **37**:99–149.
37. **Voller, A., D. Bidwell, and A. Bartlett.** 1976. Microplate enzyme immunoassays for the immunodiagnosis of virus infections, p. 506–512. *In* N. E. Rose and H. Friedman (ed.), Manual of clinical immunology. American Society for Microbiology, Washington, D.C.
38. **Young, L. S., D. Clark, J. W. Sixbey, and A. B. Rickinson.** 1986. Epstein-Barr virus receptors on human pharyngeal epithelia. Lancet **i**:240–242.