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Virus-Lymphocyte Interactions: Inductive Signals Necessary To Render B Lymphocytes Susceptible to Vesicular Stomatitis Virus Infection

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We examined the inductive signals necessary to render B lymphocytes capable of supporting a productive vesicular stomatitis virus infection. Small murine splenic B cells in the G_0 phase of the cell cycle were cultured with stimulators which allow progression through various stages in the activation and/or differentiation pathway leading to antibody secretion. We found that vesicular stomatitis virus expression is dependent on the state of B-cell activation and that three distinct phases can be defined. A nonsupportive state, which is defined by the failure to produce infection centers, viral proteins, or PFUs, is characteristic of freshly isolated small B cells, B cells cultured 48 h without further stimulation, or B cells in the G_1 phase of the cell cycle induced by culture with T-cell-derived lymphokines. This refractory state was not due to a failure of virus uptake. Activation of G_0 B cells with anti-immunoglobulin at doses which allow entry into the S phase rendered them capable of synthesizing viral proteins and increased the number of B cells producing infection centers, without enhancing PFU production on a per cell basis. In contrast, B cells stimulated with multiple inductive signals provided by anti-immunoglobulin and lymphokines showed increased infectious particle production (7 PFU per infection center). Lipopolysaccharide stimulation, acting through another induction pathway, caused the maximum increase in the number of infected B cells and production of infectious particles (25 PFU per infection center).

The alarming increase in virus-mediated immunodeficiency diseases has focused attention on the interactions between viruses and the immune system under conditions where lymphocytes and monocytes serve as targets for virus infection. Potential restrictions on these interactions can be imposed by unique features of the virus and/or intrinsic properties of the target cell that may alter susceptibility. As host cells, lymphocytes can present significant impediments to virus infection.

The availability of receptors for virus binding and entry is the first potential level of restriction. The necessary receptor may be confined to a lineage, to a subpopulation within a lineage, or to cells in a particular activation state. For example, B lymphocytes are the primary target of Epstein-Barr virus, because they are the principle cell population that expresses the CR2 (CD21) surface antigen, which is used as a receptor for virus entry (8, 9). B cells stimulated to enter the S phase of the cell cycle lose their susceptibility to Epstein-Barr virus because of down regulation of the CR2 molecule from the cell surface (6, 10, 38).

The necessity to induce critical biosynthetic pathways in the target cell provides a second level of restriction for virus infection, since most viruses are dependent for their replication on cellular factors that may be limited to cells in a particular activation state or are expressed only in cells in a particular state of differentiation. Normally, T and B lymphocytes, both sessile and recirculating, are in the metabolically quiescent (G_0), resting phase of the cell cycle; in this state, the cells have little cytoplasmic volume (35), minimal biosynthetic capability (17, 34), and are poor hosts for viral infection (4, 18, 31). Upon activation, lymphocytes become more biosynthetically capable of supporting the synthesis of viral proteins and nucleic acids, as illustrated by the finding that mitogenically stimulated lymphocytes will support a virus infection that resting B and T cells cannot (4, 15, 25, 41).

Although the requirement for "activation" is well known, the actual activation requirements for a productive infection are not. For B cells, the process of activation is complex; mitogens, specific antigen, lymphokines, or polyclonal activators such as anti-immunoglobulin, although acting through different pathways (13), are each capable of driving, to various extents, resting B cells through the differentiation pathway towards antibody secretion. Thus, exposure to different inductive stimuli provides the opportunity to correlate the ability of a B cell to support a productive virus infection with the distinct cellular changes induced by an activating agent.

We are interested in determining the spectrum of inductive signals that render B lymphocytes susceptible to virus infection. This analysis is facilitated by the ability to prepare a homogeneous starting population of resting small B cells in the G_0 phase of the cell cycle for analysis. Activation of a small B cell results in a dramatic increase in the biochemical activity of the cell, with a concurrent increase in cell size and decrease in cell density (13, 34). The physical changes in activated B cells allow for the fractionation of freshly isolated cell populations into small resting or large activated B cells by bouyant density and sedimentation velocity centrifugation. We determined how the process of infection is affected by the activation process by assessing susceptibility to virus infection of resting B-cell populations exposed to defined activation inducers that were chosen on the basis of the following properties: (i) exposure of small resting B cells to anti-immunoglobulin plus lymphokines derived from T-helper cells or to the mitogen lipopolysaccharide (LPS) allows full activation, causing both proliferation and differentiation to antibody-secreting cells (17, 33); (ii) incubation

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with anti-immunoglobulin causes small resting G_0 B cells to enter the S phase without further differentiation (13); and (iii) lymphokines, in particular interleukin-4 (IL-4), support B-cell transit into the G_1 phase of the cell cycle (30, 34).

We analyzed changes in B-cell susceptibility to infection by using vesicular stomatitis virus (VSV). VSV is a negative-strand RNA virus in the rhabdovirus family. Replication occurs in the cytoplasm of the infected cell and is dependent upon host cell factors (41). We followed the process of infection experimentally in B-cell populations by (i) using sodium dodecyl sulfate gel electrophoresis of [^{35}]methionine-labeled cell lysates to ascertain viral protein synthesis, (ii) determining the number of infected cells in the population by an infection center formation, and (iii) determining the titers of infectious virus particles in the supernatants of infected cultures.

We found striking differences in the ability of B cells to support viral protein synthesis and/or production of infectious virus particles which are dependent on the activation state induced. These effects are not due to a restriction on virus entry.

MATERIALS AND METHODS

Mice. CBA/NJ × A.By/J female or A.By/J × CBA/NJ male and female mice were bred and maintained in our own animal facilities and used between 12 and 24 weeks of age. Parental stocks of CBA/NJ and A.By/J were obtained from Jackson Laboratories, Bar Harbor, Maine.

Virus stock. VSV Salt Lake City was obtained from T. Morrison (University of Massachusetts Medical School), and stocks were prepared in CHO cells and purified as previously described (7).

B-lymphocyte preparation. Spleen cell suspensions were prepared by gently pressing spleens between glass microscope slides, depleting debris by passage through nylon mesh and red blood cells by treatment with Tris-buffered NH_4Cl (26). Pooled spleen cell suspensions were washed twice by centrifugation through modified Hanks balanced salt solution supplemented with 1.5% (vol/vol) fetal calf serum (BSS-FCS) and enriched for B cells by complement-mediated depletion of T cells as follows: cells were exposed to anti-Thy 1.2 and anti-Ly 2.2 monoclonal antibodies for 1 h on ice, washed, and treated for 1 h at 37°C with a 1:10 dilution of rabbit complement (Pelfreeze, Rodgers, Ark.) or guinea pig complement (Rockland, Gilbertsville, Pa.) preadsorbed on mouse splenocytes.

Cell separation. Splenocytes enriched for B cells were suspended to 50×10^6/ml in BSS-FCS supplemented with 125 μg of DNase I (Sigma Chemical Co., St. Louis, Mo.) per ml and incubated for 30 min at 22°C. The B-cell population was separated into small and large cell fractions by counterflow centrifugal elutriation (model J-6B; Beckman Instruments, Inc., Palo Alto, Calif.) as previously described (2). Cells eluting at a flow rate of 20 ml/min with a symmetrical peak and a mean cell volume of 120 μm^3 were collected and defined as the small resting fraction (37) (approximately 20% of input cells). This stringent size cut was used to ensure that the starting population was in the resting state. Spontaneous [^{3}H]thymidine incorporation was not observed in these small cell populations. Cell size was determined by using a Channelyzer (Coulter Electronics, Inc., Hialeah, Fla.). Cells eluting after a flow rate of 22 ml/min was reached contained endogenously activated B cells, macrophages, and polymorphonuclear monocytes.

B-cell cultures. Small B cells were cultured in complete medium (CM) as follows: RPMI 1640 supplemented with 10% fetal calf serum–2 mM L-glutamine per ml–100 U of penicillin per ml–100 μg of streptomycin per ml–10 μg of gentamicin per ml–1× minimal essential medium nonessential amino acids–5×10^{-5} M 2-mercaptoethanol per ml. Cells were cultured with various lymphokines and mitogens in 60-mm (diameter) tissue culture dishes, 5 ml per dish at a cell concentration of 5×10^6/ml at 37°C in a 5% CO_2–air mixture.

Cell proliferation in B-cell cultures activated by mitogens and/or lymphokines was assayed by [^{3}H]thymidine uptake. Cultured B cells or hybridoma cells were harvested and washed, and triplicate samples of 5×10^5/200 μl of viable cells were placed into 96-well tissue culture dishes with 1 μCi per well of ^{3}H-thymidine (NET-027 [6.7 Ci/mM]; DuPont, NEN Research Products, Boston, Mass.). Plates were incubated at 37°C for 6 h and harvested by using a PHD cell harvester (Cambridge Technology Inc., Cambridge, Mass.), and thymidine incorporation was assessed by scintillation counting.

Mitogens and lymphokines. The B-cell mitogen, LPS (Bacto Lipopolysaccharide *E. coli* 055:B5 [Westphal method]; Difco Laboratories) was used at a final concentration of 50 μg/ml of culture medium. Rabbit anti-mouse $F(ab')_2$ antiserum (anti-immunoglobulin) was obtained from rabbits hyperimmunized with $F(ab')_2$ fragments of monoclonal antibody UPC10 (IgG_1,κ). Anti-$F(ab')_2$ antibodies were affinity purified from hyperimmune serum by adsorption and acid elution from an affinity column of normal mouse immunoglobulin coupled to Sepharose 4B. $F(ab')_2$ fragments of the rabbit antibody were prepared by digestion with pepsin followed by chromatography on a Sephadex G-100 column and used as a B-cell mitogen at 10 to 25 μg/ml. Lymphokine-containing supernatants (SN) were prepared from cloned lines of distinct T-helper-cell subpopulations; Th_1 clone D1.6 (21) and Th_2 clones CDC 25 or CDC 35 (37) were generously provided by D. Parker, University of Massachusetts Medical School, by induction for 24 h with 4 μg of concanavalin A (Sigma) per ml at a cell concentration of 10^6/ml. Lymphokine-containing SN was also prepared by incubating total spleen cells from A.By/J or A/J mice at a cell concentration of 10^7/ml with 4 μg of concanavalin A per ml for 24 h. Concanavalin A was removed from all SN by passage over a methyl-α-D-mannopyranoside–Sepharose column. All SN were used at a 50% concentration in cultures supplemented with methyl-α-D-mannopyranoside at 10 mg/ml.

Antibodies and cell lines. The following monoclonal antibodies were used in depletion experiments: anti-Thy 1.2 (ATCC TIB 99) (23), anti-Ly 2.2 (ATCC TIB 150), anti-B220 (14.8) (20), RA3.3A1 (8), anti-Ia^k (14.4.4S) (32), 10.2.16 (ATCC TIB 93), and 11.5.2 (ATCC TIB 94), anti-B cell (J11D) (5), anti-rat kappa (Mar 18.5) (ATCC TIB 216). Also, the B-cell hybridoma fusion partner Sp2/0 (ATCC CRL 1581) or the antibody-secreting hybridoma 36-65 (24) were found to be permissive for virus infection and were used as control susceptible B-cell target lines in all experiments. Rabbit anti-VSV serum, generously supplied by T. Morrison, was used to neutralize virus.

Assays for virus infection. B cells, usually 5×10^6 cells cultured for various times under various activation conditions, were infected with VSV at a multiplicity of infection of 10 PFU per cell for 1 h at 37°C. Cells were washed twice with CM and resuspended in CM with sufficient rabbit anti-VSV serum to neutralize all input virus. After 30 min at 37°C, cells were washed twice, suspended to the original volume, and counted. Samples were also taken for an infection center

assay, an overnight culture for determining virus production, and for [^{35}S]methionine labeling of viral and host proteins.

The number of infected cells was determined by using an infection center assay. Briefly, duplicate 0.2-ml samples of serially diluted cells were plated onto 60-mm (diameter) tissue culture dishes of secondary chick embryo fibroblast monolayers. Cell samples were plated in a 1:1 mix of CM containing cells and overlay medium (minimum essential medium with penicillin, streptomycin, gentamicin, tryptose phosphate broth, $CaCl_2$, 0.8% agarose [Seaplaque; FMC Corp.], and 0.2% agar [Bacto-Agar; Difco]). After 1 h, the monolayers were overlaid with an additional 5 ml of overlay medium. Virus plaques were counted at 24 and 48 h.

Virus production (PFU) was assayed 24 h after infection by incubating 5×10^5 infected cells in 1 ml of CM overnight in 24-well tissue culture dishes. SN were collected and titers of infectious virus were determined on secondary chick embryo fibroblast monolayers.

In some experiments, small B cells were preinfected with VSV at a multiplicity of infection of 10 for 2 h at 37°C and washed, and excess virus was neutralized before activation in culture. After 24 or 48 h in culture, these preinfected cells were washed and excess virus was again neutralized before analyzing these cells for infection center formation and virus production.

Viral protein synthesis was examined by continuous labeling with [^{35}S]methionine for 2 h of cells infected 8 to 10 h previously. Briefly, cells were washed twice in CM made with methionine-free RPMI 1640, suspended at 10×10^6/ml with 50 μCi of [^{35}S]methionine (SJ-1515; specific activity, 1,250 Ci/mM; Amersham Corp., Arlington Heights, Ill.) and pulsed for 2 h at 37°C. Thirty minutes prior to cell lysis, iodoacetamide (Sigma) in phosphate-buffered saline was added to a final concentration of 7 to 10 mM. Cells were spun down, SN was removed, and cell pellets were lysed with lysing buffer (0.5% Nonidet P-40, 0.5% deoxycholate, 50 mM Tris [pH 8.0]) with phenylmethylsulfonyl fluoride (final concentration, 2 mM). Cell lysates were microfuged, and the SN was analyzed on a 10% sodium dodecyl sulfate–acrylamide gel with fluorography.

Immunoprecipitation of viral proteins was carried out by adding protein A-Sepharose 4B beads (Sigma) previously loaded with rabbit anti-VSV antibodies for 2 h at room temperature and then washed with 0.5% Nonidet P-40 in 50 mM Tris (pH 8.0) to cell lysates. Samples were incubated for 2 h at room temperature and washed four times with 0.5% Nonidet P-40–400 mM NaCl in 50 mM Tris (pH 8.0), and radiolabeled proteins were eluted by boiling in SDS-gel sample buffer for 5 min and electrophoresed on a 10% SDS–acrylamide gel.

RESULTS

VSV infection in B-cell-enriched spleen cell populations. The ability of murine spleen cell populations enriched for B cells to support VSV infection and replication after activation with various stimuli was determined. The B cells used for these experiments were freshly isolated and unelutriated and represented a continuum of activation states due to in vivo exposure to environmental antigens. Cells so prepared are enriched for B cells as indicated by the >95% fluorescent staining for surface immunoglobulin (J. Riggs, personal communication). Table 1, experiment 1, shows the number of infection centers per 10^3 plated cells exposed to different culture conditions for 48 h. B cells cultured for 48 h with no additional stimulus produced 39 infection centers per 10^3 cells, considered here as the unactivated background response. Cells cultured with splenic SN factors, which support transition to the G_1 phase of the cell cycle, produced a modest increase in infection centers, while B cells which received inductive signals from anti-immunoglobulin, which allows transit to the S phase produced 200 infection centers. Cells which were exposed to more rigorous stimulation with anti-immunoglobulin plus splenic SN or LPS produced more infection centers (340 and 300, respectively, per 10^3 cells). Thus, up to 30% of the cells activated with anti-immunoglobulin plus SN or LPS can support virus infection which is comparable by this assay to that observed in the permissive cell lines CHO (21%) and SP2/0 (34%). Experiment 2 and others not shown yielded similar results.

TABLE 1. B-cell-enriched spleen populations are targets for VSV infection[a]

Stimulus	Mean infection centers/10^3 cells plated[b]	
	Expt 1	Expt 2
None	39 (11)	ND[c]
Splenic SN	90 (26)	45 (21)
Anti-immunoglobulin	200 (58)	208 (99)
Anti-immunoglobulin + SN	340 (98)	277 (131)
LPS	300 (87)	165 (78)

[a] B-cell-enriched splenocyte populations were cultured with the indicated stimuli for 48 h before virus infection.

[b] Mean of duplicate determinations averaged over three serial dilutions. Values in parentheses are percentages of the control values determined by using permissive cell lines. In experiment 1, SP2/0 gave 344 infection centers per 10^3 cells plated; in experiment 2, CHO cells gave 210 infection centers per 10^3 cells plated.

[c] ND, Not done.

These data also imply that B cells are the target for VSV infection, given that 95% of the starting cell population is sIg^+, and given the specificity of B-cell induction with anti-immunoglobulin and LPS activators. Moreover, because not all B cells are activated to proliferation by LPS or anti-immunoglobulin (30 and 60%, respectively [1, 14]), these results also suggest that a very high proportion of activated cells are infected.

Small resting B cells can be activated to support VSV. Although they established the target population, the experiments described above could not define the minimum activation signal(s) required for virus infection, because of the heterogeneous nature of the B cells in the starting population. Accordingly, subsequent assessments of the role of activation employed freshly isolated elutriated small B cells. This population was unable to support virus infection following exposure to VSV, as assessed by infection center assay (4 infection centers per 10^3 plated cells) or virus production (Table 2, experiment 1). Moreover, B cells maintained in culture for 48 h without stimulation also showed no increase in the number of infection centers ($3/10^3$ plated cells), thus demonstrating that no inducers are found in the culture medium. This small number of infection centers produced by freshly isolated or cultured but unstimulated small B cells is in striking contrast to that found for the unstimulated total B-cell-enriched population (Table 1). This change is likely the result of the removal of endogenously activated B cells or other VSV supportive cells from the elutriated small B cell population.

Infection center formation markedly increased in small B cells exposed to B-cell activators. In this regard, LPS activation was the most effective; at 48 h, there was a 65-fold increase over the small B-cell background to 195 infection

TABLE 2. Activation of small resting B cells to support VSV infection[a]

Stimulus	Mean infection centers/10^3 cells plated[b]	PFU/10^3 cells cultured	PFU/IC[c]
Expt 1			
None[d]	4	1	<1
None (cultured 48 h)	3	2	<1
Anti-immunoglobulin	90	52	<1
Anti-immunoglobulin + splenic SN	62	450	7
LPS	195	5,300	27
Expt 2			
Anti-immunoglobulin	ND	51	
Anti-immunoglobulin + Th_2 SN	ND	395	
LPS	ND	2,050	

[a] Elutriated small B-cell populations were cultured with the indicated stimulus for 48 h before virus infection. Experiments 1 and 2 used different starting populations. As a positive control, 36-65 hybridoma, freshly isolated from culture, gave 339 infection centers per 10^3 cells and 1.9×10^4 PFU/10^3 cultured cells in experiment 1 and 1.4×10^4 PFU/10^3 cultured cells in experiment 2.

[b] Mean of duplicate determinations averaged over three serial dilutions. ND, Not done.

[c] Number of infectious particles per infection center (IC).

[d] Elutriated small B cells exposed to VSV on the day of isolation.

centers per 10^3 cells plated; exposure to anti-immunoglobulin with or without supplementation with lymphokines caused 20- to 30-fold increases, respectively.

The most striking change brought about by a variation in activation stimulus was in the production of infectious particles. B cells stimulated with anti-immunoglobulin increased the number of infection centers (90/10^3 cells plated) and increased the PFU produced (52/10^3 cells cultured), but the virus yield on a per cell basis was less than 1 PFU per infection center, which is comparable to that seen in unstimulated B-cell cultures. In contrast, anti-immunoglobulin plus SN increased PFU formation 200-fold overall and also increased virus production on a per cell basis to 7 PFU per infection center. LPS-stimulated B-cell cultures were most efficient in PFU production, which increased 2,000-fold over the small B-cell background to 25 PFU per infection center, a value close to the level of virus production seen in permissive cell lines. In other experiments, B cells were cultured with anti-immunoglobulin and SN from Th_2 T-cell clones (Table 2, experiment 2). While these SN contain a more restricted array of growth-promoting lymphokines than splenic SN (27), virus production was comparable to that observed in anti-immunoglobulin plus splenic SN cultures. These data demonstrate that both the number of cells infected and the level of PFU production are affected by the activation stimuli.

The progress of infection was also monitored by examining viral protein synthesis. As expected, VSV proteins were readily seen in productively infected B cells stimulated by anti-immunoglobulin and SN or LPS (Fig. 1, lanes 4 and 8). VSV protein synthesis was also seen in cells stimulated with anti-immunoglobulin, despite the fact that low numbers of infectious particles were produced (Fig. 1, lane 2). Immunoprecipitation shows that the lack of PFU production in anti-immunoglobulin-treated B cells is not readily correlated to qualitative differences in the array of viral proteins synthesized.

The high proportion of B cells in the starting population

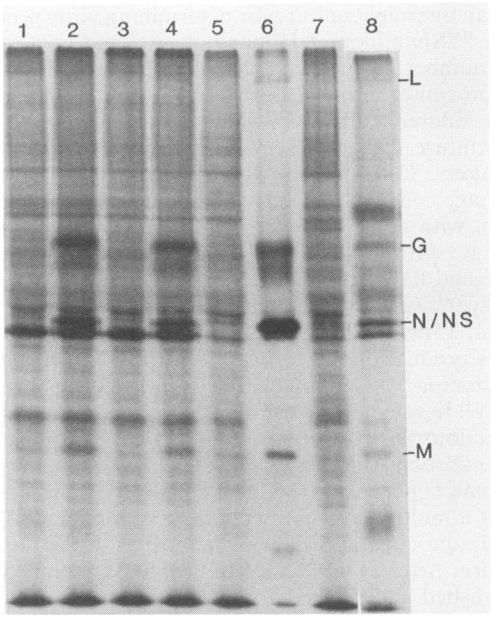

FIG. 1. Immunoprecipitation of cell lysates with rabbit anti-VSV serum from uninfected (lanes 1, 3, and 5) and infected (lanes 2, 4, 6, 7, and 8) cells treated with anti-immunoglobulin (lanes 1 and 2), anti-immunoglobulin plus splenic SN (lanes 3 and 4), Sp2/0 cell line (lanes 5 and 6), splenic SN (lane 7) and LPS (lane 8). At right are position markers for the following viral proteins: RNA polymerase (L), glycoprotein (G), nucleocapsid-nonstructural protein (N/NS), and membrane protein (M).

combined with the specificity of the inducers for B cells and the high number of infected cells in the cultures is consistent with the notion that B cells are the target of virus infection in this system. This position is directly supported by experiments where B-cell-specific monoclonal antibodies were used with complement to deplete VSV-infected B-cell populations previously activated with anti-immunoglobulin and SN. Treatment with anti-B220, anti-Ia^k, or J11D resulted in a decrease in cell numbers of 37, 88, and 78%, respectively, of cultured cells, with a corresponding decrease in infection centers of 44, 86, and 65%, respectively. PFU production also decreased (data not shown). These data demonstrate, by a number of criteria, that B cells are the target population and activation renders them susceptible to infection.

Multiple signals are required for a productive VSV infection. All B-cell inductive regimens employed in previous experiments allowed progression of the B cells into the initial S phase of the cell cycle, as indicated by the incorporation of [^{3}H]thymidine (Fig. 2, bars 5 to 9). Yet the data for anti-immunoglobulin-stimulated cells show that entry into the S phase is not sufficient for a productive VSV infection. However, B cells cultured with anti-immunoglobulin and lymphokines did show PFU production, suggesting that either additional differentiation steps supported by lymphokines are necessary for virus production or that lymphokines can directly activate B cells to a permissive state without other inductive signals. Indeed, lymphokines facilitate the G_0 to G_1 cell cycle transit in small B cells, causing increases in B-cell volume, RNA synthesis, and protein synthesis (30, 36), which may be sufficient to support virus infection. To examine this possibility, we cultured small B cells with lymphokine-containing SN and determined their subsequent susceptibility to VSV infection. Lymphokines were derived from SN of concanavalin A-activated splenic T cells or

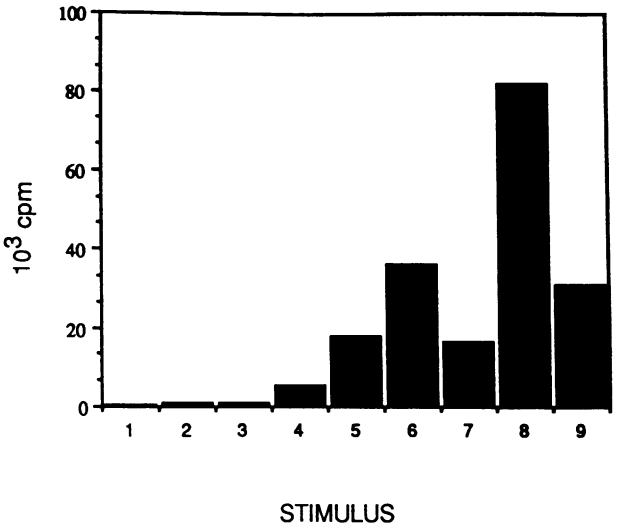

FIG. 2. [^{3}H]thymidine incorporation in cultured B-cell populations. Treatment: Bar 1, unstimulated; bars 2 to 9, stimulation with splenic SN (bar 2), Th_1 SN (D1.6) (bar 3), Th_2 SN (CDC 35) (bar 4), anti-immunoglobulin (bar 5), anti-immunoglobulin plus splenic SN (bar 6), anti-immunoglobulin plus Th_1 SN (bar 7), anti-immunoglobulin plus Th_2 SN (bar 8), and LPS (bar 9).

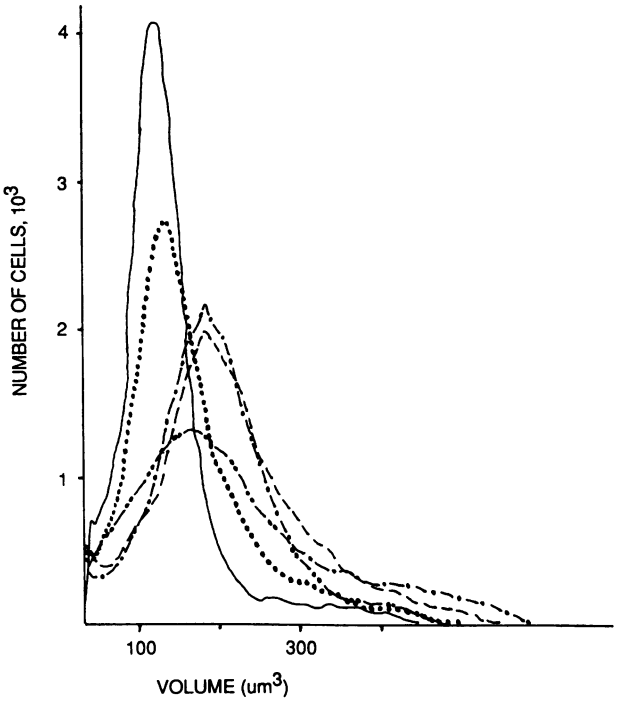

FIG. 3. Cell volume profiles of elutriated B-cell populations. Shown are results for freshly isolated small B cells (——) or populations cultured for 48 h unstimulated (·····) or stimulated with splenic SN (—·—), Th_1 SN (D1.6) (– –), or Th_2 SN (CDC 35) (—··—).

T-helper cell clones. T-helper cell clones represent two distinct T-helper cell subpopulations (Th_1 and Th_2) (27). Th_1 cells secrete IL-2 and gamma interferon and Th_2 cells secrete IL-4, IL-5, and IL-6 as their unique lymphokines, whereas both secrete IL-3, lymphotoxin, and granulocyte-macrophage colony-stimulating factor. Small B cells exposed to these SNs for 48 h produced no infection centers, PFUs (Table 3), or viral proteins (Fig. 1, lane 7 [splenic SN]). The SNs did induce the expected increase in B-cell size (Fig. 3) and protein synthesis, as indicated by a 10-fold increase in [^{35}S]methionine incorporated into trichloroacetic acid-precipitable material (data not shown). Thus, lymphokine exposure alone is not sufficient to render B cells capable of supporting VSV infection, suggesting the requirement for multiple activation signals.

VSV entry into resting B cells. Our previous findings showed that freshly isolated, unstimulated but cultured, or lymphokine-treated B cells were incapable of supporting VSV infection, as assessed by our criteria for infection. Since this could suggest that activation was necessary for virus entry, we next determined if nonpermissive B cells were blocked at this level. Initial experiments using ^{35}S-labeled virions to examine VSV particle uptake by freshly isolated or unstimulated, cultured small resting B cells indicated no apparent block at either binding or internalization when compared with control populations (data not shown). Based upon these studies, we used a second approach which assumed that small B cells exposed to VSV could be made to express virus when activated to a permissive state. This also assumes that the input virus is not degraded in the cell before activation. Accordingly, small resting B cells were incubated with VSV and excess virus was neutralized. Cells so treated were cultured without stimulation or were exposed to splenic SN or LPS for 24 and 48 h. A representative experiment using this approach is shown in Table 4, experiment 1. At 24 h, there was no significant increase in infection foci in any population which demonstrated the requirement for B-cell activation for virus expression. By 48 h, there was a marked increase in the number of infection centers in the LPS-stimulated cultures (122 per 10^3 plated cells) as well as PFU production (1,700 per 10^3 cells cultured), values which are comparable to those obtained when B cells were activated with LPS before VSV exposure (Table 4, experiment 2). In contrast, unstimulated B cells or B cells exposed to lymphokines remain nonpermissive. Thus, the ability of B cells to support VSV infection is dependent upon cellular changes associated with B-cell activation and is not due to a failure in virus uptake.

TABLE 3. Inability of small B cells stimulated with lymphokine-rich SN to support VSV infection[a]

Stimulus	Mean infection centers/10^3 cells plated[b]	PFU/10^3 cells cultured	PFU/IC[c]
None[d]	1	4	4
Splenic SN	3	8	3
Th_1 SN (D1.6)	4	2	<1
Th_2 SN (CDC 35)	6	12	2
Anti-immunoglobulin	110	58	<1
LPS	330	8,560	26

[a] Elutriated small B cells were cultured with the indicated stimulus for 48 h before virus infection.
[b] Mean of duplicate determinations averaged over three serial dilutions.
[c] Number of infectious particles per infection center (IC).
[d] Cells cultured for 48 h without additions.

DISCUSSION

The experiments presented here define at least three distinct phases during B-cell activation that support VSV infection to various extents and suggest that optimum virus production is critically dependent on host-derived matura-

TABLE 4. Infection center formation and virus production in small resting B cells following VSV exposure and in vitro culture

Stimulus[a]	Mean infection centers/10^3 cells plated[b]		PFU/10^3 cells cultured for 48 h	PFU/IC[c]
	24 h	48 h		
Expt 1				
None	2	5	15	3
Splenic SN	3	10	11	1
LPS	11	122	1,700	14
Expt 2				
None		6	9	1
Splenic SN		5	6	1
LPS		152	3,815	25

[a] In expt 1, small B cells were infected on the day of isolation and then cultured for 24 or 48 h with the indicated stimulus before assay. In expt 2, another sample from the same population of small B cells was cultured for 48 h with the indicated stimulus prior to VSV infection.
[b] Mean of duplicate determinations averaged over two serial dilutions.
[c] Number of infectious particles per infection center.

tion factor(s) produced in B cells undergoing the activation process.

Our finding that murine B cells can be productively infected by VSV is in contrast to the work of other investigators (3, 4, 19, 39) who have previously reported that spleen B-cell populations, partially purified by different techniques, exposed to LPS remain refractory to infection. That B cells are the major targets of VSV in our system is demonstrated by a number of lines of evidence. First and foremost is the relative purity of our target cell population, which is greater than 95% surface immunoglobulin positive. Further, purification of small B cells by countercurrent elutriation and the use of LPS or anti-immunoglobulin served to selectively activate only B cells in the starting population. Moreover, culture of the starting small B-lymphocyte population with lymphokines in the absence of anti-immunoglobulin failed to induce cells capable of supporting viral protein synthesis or the production of infectious virus particles, thus excluding the possibility that a quiescent contaminating cell population is enriched or activated by lymphokine exposure. We also found that B-cell-specific monoclonal antibodies in the presence of complement can specifically deplete up to 88% of the cells giving rise to infectious foci and to infectious particles in VSV-infected B-cell populations activated in culture with anti-immunoglobulin and T-cell-derived lymphokines.

Virus infection in activated B cells is efficient. In B cell populations cultured with LPS or anti-immunoglobulin and lymphokines, the relative proportion of susceptible cells, as monitored by infection center formation, was comparable to the fraction of B cells known to be stimulated to proliferation under the conditions used in these experiments (1, 14). Moreover, virus production, as monitored by determining the titers of infectious particles, was similar to that found when conventional VSV-susceptible target populations, such as CHO cells, were analyzed.

Activation is clearly necessary to render B cells susceptible to infection, as small resting B cells in the G_0 stage of the cell cycle are refractory to VSV. This refractory state is not due to the absence of a critical receptor necessary for virus entry. We have shown that small lymphocytes previously exposed to VSV will go on to produce infectious foci and infectious virus particles upon subsequent exposure to LPS. Thus, a small B cell can take up virus but requires activation to support the process of infection.

What cellular changes constitute a supportive state? Exposure to lymphokines from cloned Th_1 or Th_2 cells or polyclonally activated splenic T cells, which cause transit from G_0 to G_1 and B-cell enlargement (Fig. 2), was not sufficient to render B cells capable of supporting VSV or even the production of virus proteins. Because preliminary experiments indicate that these cells produce all VSV mRNAs, the block is not at the level of primary viral transcription (M. R. Schmidt, K. A. Gravel, and R. T. Woodland, Program Abstr. ICN-UCI Int. Conf. Virol., abstr. no. 41, 1990). We are currently trying to determine if lymphokine exposure is neutral or renders the cell anergic to inductive stimuli that would normally support a productive virus infection.

Small B cells cultured with mitogenic doses of anti-immunoglobulin produced viral proteins and showed a 30-fold increase in infection centers over background but failed to produce infectious virus particles at a high rate (less than one per infection center). This apparent disparity between the number of infection centers and PFUs detected in SN may be owing to the potential for readsorption of released virus particles by B cells in liquid culture. In contrast, virus production as assayed by focus formation would be expected to be more unambiguous because of direct contact of an infected B cell with the susceptible monolayer. Anti-immunoglobulin-stimulated B cells have some characteristics of persistently infected cells which also produce no or low levels of infectious particles. The restrictions on the production of infectious particles in anti-immunoglobulin-treated small B cells are similar to restrictions seen for high-rate immunoglobulin synthesis and secretion, as exposure to lymphokines is necessary to facilitate both processes. Thus, neither lymphokines nor anti-immunoglobulin alone was sufficient to render B cells capable of high-level production of infectious particles.

Activation of B cells with anti-immunoglobulin plus lymphokines results in a fully permissive cell population with a 10-fold increase in production of infectious virus particles per infection center over that seen with B cells treated with anti-immunoglobulin alone. These results are consistent with a requirement for at least two inductive signals to render B cells supportive of VSV production. Anti-immunoglobulin mediates receptor cross-linking and internal signalling followed by the induction of new lymphokine receptors which allow subsequent differentiation events supported by lymphokine(s) present in the T-cell-derived SN and leads to increased virus production. One aspect of these results is paradoxical. The concanavalin A-activated spleen cell SN used as one source of lymphokines contain gamma interferon at levels sufficient to inhibit VSV production by infected L cells (R. Welsh, personal communication). This may suggest that gamma interferon, which facilitates the maturation of activated B cells and the expression of the IgG2a immunoglobulin isotype (34) stimulates minimal antiviral activity on the B cells activated with anti-immunoglobulin. It has been reported that gamma interferon acts as a growth factor rather than an antiviral factor for activated T cells (22). Alternatively, our finding that SN prepared from Th_2 clones, which secrete no gamma interferon, facilitates VSV production in anti-immunoglobulin-treated B cells as well as but no better than spleen cell SN (Table 2) may suggest that the response to enhancing lymphokine(s) in splenic SN can counteract potentially suppressive effects due to interferon or other cytokines. In this regard, it is noteworthy that IL-4 and gamma interferon have antagonistic effects on immunoglobulin isotype expression (30).

LPS is the most effective inductive regimen for activating B cells to support VSV. Infectious virus production per cell is comparable to that seen with permissive cell lines, such as CHO and SP2/0 (Table 1) and threefold over that seen in anti-immunoglobulin-plus-lymphokine-treated cultures. Although different pathways of activation are utilized by these two classes of activators (13), both activation sequences result in differentiation to antibody production, suggesting that subsequent cellular changes during B-cell activation ultimately determine the level of PFU production. LPS-activated B cells also show another property. Preliminary experiments indicate that exposure to spleen cell-, Th_1-, or Th_2-derived lymphokines during LPS activation suppresses VSV PFUs to background levels (M. R. Schmidt et al., Abstr. Annu. Meet. Am. Soc. Virol. 1990), thus demonstrating a completely different response pattern to the same collection of lymphokines from that of B cells activated with anti-immunoglobulin. This reinforces the notion of enhancing and suppressive lymphokines in the same SN and suggests to us a number of possible models to explain the behavior of B cells exposed to these lymphokines. B cells reactive to LPS or anti-immunoglobulin may represent stable and distinct B-cell subpopulations (1, 16) which differ in the lymphokines to which they respond or respond differently to the same lymphokines. Alternatively, LPS and anti-immunoglobulin may both act on a single B-cell population, but each inducer, acting through a distinct activation pathway, causes a different array of lymphokine receptors to be expressed or modifies the potential of the B cell to respond to the same collection of lymphokines. Additional studies are in progress to distinguish among these possibilities.

Restrictions on VSV expression in transformed lymphoblastoid cell lines have been reported previously (11, 12, 28, 29, 40). However, it is not always possible to determine how the process of transformation may alter the course of virus expression as it would occur in normal cells at equivalent stages of differentiation or activation. In this report, we describe a system to correlate the biochemical changes occurring during the activation of normal B cells with the ability to support a productive virus infection. In addition, as many lymphokines have been cloned and are available as recombinant molecules, this system permits another level of analysis of the cellular changes induced by individual lymphokines or combinations of lymphokines which may not have been detected by previous methods.

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