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Virus-Lymphocyte Interactions: Virus Expression Is Differentially Modulated by B Cell Activation Signals: A Dissertation

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VIRUS-LYMPHOCYTE INTERACTIONS:

VIRUS EXPRESSION IS DIFFERENTIALLY MODULATED

BY B CELL ACTIVATION SIGNALS

A Dissertation Presented

By

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ABSTRACT

It is shown here that the ability of B lymphocytes to act as supportive host cells for virus infections requires they be activated from the resting G_o stage of the cell cycle. I have used a series of activation regimens, which allow B cells to progress to different stages in their activation/differentiation pathway toward antibody secretion, in order to evaluate the extent of activation required to support vesicular stomatitis or Newcastle disease virus infections.

At least three distinct phases during B cell activation which affected VSV infection were defined. Freshly isolated resting murine splenic B cells in the G_o phase of the cell cycle do not support VSV, assessed by protein synthesis, infectious center formation, and PFU production. Small B cells cultured for 48 hours without stimulation still do not support VSV. B cells stimulated with the lymphokines found in Con A activated supernatants from splenic T cells or cloned T cell lines transited into the G_1 phase of the cell cycle but remain refractory to VSV. These VSV non-supportive B cell populations do take up virus particles and transcribe viral mRNAs which can be translated <u>in vitro</u>, suggesting a translational block to

VSV. B cells stimulated into the S phase of the cell cycle with anti-immunoglobulin synthesize VSV proteins and increased numbers of infectious centers, but only low level PFU synthesis (<1PFU per infectious center) is observed. Co-stimulation with anti-Ig and lymphokines, which supports differentiation to antibody secretion, enhanced PFU synthesis without further increasing the number of infected B cells. LPS, which activates B cells directly to antibody secretion by a pathway different from anti-Ig, induced infectious centers, and PFUs at levels comparable to those seen when stably transformed permissive cell lines are infected. Co-stimulation of LPS activated B cells with the same lymphokine populations that enhance PFU production when anti-Ig is used as a stimulator suppresses PFU production completely, suggesting that anti-Ig and LPS activated B cells are differentially responsive to lymphokines.

NDV infection of murine B cells differed markedly from VSV infection, as all B cell populations examined gave a similar response pattern. NDV viral proteins were synthesized by B cells in each of the activation states previously described, even freshly isolated B cells. Infectious center formation increased up to 5-fold over

the levels observed with unstimulated B cells after anti-Ig or LPS activation. However, PFU synthesis was low (<1 PFU per infectious center) for all B cell populations.

These results suggest that these two similar viruses may be dependent on different host cell factors and that these factors are induced for VSV but not NDV by the B cell activators employed here or that the process of infection of B cell by these two viruses induces different cellular responses.

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INTRODUCTION

Cells of the lymphoid lineage can be host cells for infection by many viruses of all taxonomic groups: RNA and DNA genomes, cytoplasmic and nuclear replication patterns (review: McChesney and Oldstone, 1987). Suppression of immune system function is observed coincidental with many acute viral infections in man and animals, demonstrating one important consequence of infection of immune system cells. Immune suppression may have more physiological consequences for the host than the infection of other organs and tissues, as it permits the reactivation of other pathogens or allows opportunistic infections to occur. Suppression may be direct, in that infected lymphocytes are eliminated through virus-mediated cytolysis, or indirect, whereby lymphocytes are functionally altered through the loss of key differentiated functions, such as has been shown to occur for cytotoxic T cell activity or B cell antibody synthesis (Rice et.al., 1984; Casebolt et.al., 1987; Romano et.al., 1977; McFarland, 1974; Kleinerman et.al., 1974; Whittle et.al., 1978). Suppression of immune responsiveness during virus infection could be the consequence of either of these mechanisms. The focus of this dissertation is to

understand the events leading to viral parasitism of lymphocytes, to understand how the process of infection procedes and what unique aspects of lymphocyte biology serve to enhance or suppress the viral infection.

One can envision a number of ways in which the particular physiology of lymphocytes can affect their susceptibility as host cells for virus infection. The ability to enter the target cell is a first requisite for virus infection. Entry can be achieved both by specific receptor mediated endocytosis or by non-specific endocytotic events. The few virus receptors that have been well characterized are cellular proteins involved with other critical cell functions, for example: human immunodeficiency virus predominately uses the CD4 molecule on T lymphocytes for virus entry, a molecule critical for MHC class II restricted cell:cell interactions (Fauci, 1988); Epstein-Barr virus uses the complement receptor CR2 (CD21), a molecule normally involved in B cell activation (Cooper et.al., 1988); and cellular adhesion molecules, such as ICAM-1 and other members of the immunoglobulin superfamily of proteins, are receptors for rhinovirus, poliovirus, and foot and mouth disease virus (Greve et.al., 1989; Staunton et.al., 1989; Mendelson et.al., 1989; Acharya et.al., 1989). Therefore, the presence of

receptors is a major determinant of cell tropism for particular viruses.

An additional level of virus restriction involves the susceptibility of the virus receptor to modulation. Is the molecule constitutively expressed on a lymphocyte or does expression vary with the activation state? Lymphocytes are normally found in a continuum of activation states due to ongoing responses to environmental antigens or infection. Before activation both B and T lymphocytes are found as small cells in the resting (G_o) stage of the cell cycle, but following antigen exposure they are driven into the G_1 and S phases of the cell cycle, causing increased cell size, increased RNA and protein synthesis, and concurrent changes in expression of cell surface antigens. Expression of a virus receptor may therefore vary with the activation state of the lymphocyte, which could not only determine the presence or absence of a particular molecule, but also its level of expression. Successful receptor mediated endocytosis of a virus particle leading to infection may require that a virus engage simultaneously a number of receptor molecules on the host cell surface. That the level of receptor expression is important and correlated to a lymphocyte's activation state is illustrated by the

finding that the EBV receptor, present on resting B cells, is down regulated from the B cell surface upon activation to S phase of the cell cycle, making activated B cells less susceptible to infection (Casali and Notkins, 1989). Expression of a virus receptor may also be restricted to particular cell types or subpopulations within a cell type. For example, the CD4 receptor molecule for HIV is expressed only on a subpopulation of T lymphocytes. Therefore, both the presence of a receptor as well as the level of expression can affect the ability of a virus to infect lymphocytes.

A further restriction on virus infection is imposed by the physiology of lymphocytes. It has been repeatedly demonstrated that the ability of a lymphocyte to function as a host cell for a "productive" virus infection requires activation from the resting state (reviews: Wheelock and Toy, 1973; Bloom et.al., 1976; McChesney and Oldstone, 1987). For example, human peripheral blood lymphocytes required mitogenic stimulation to support measles virus infection (Huddlestone et.al., 1980). Experiments done by Lucas studying measles virus infection of human lymphocytes (Lucas et.al., 1978) and Romano studying vesicular stomatitis virus (VSV) infection of mouse lymphocytes

(Romano et.al., 1977) attempted to show that the requirement for activation was not at the level of virus entry as unstimulated lymphocytes exposed to virus produced infectious centers and PFUs upon subsequent mitogenic activation. However, the ability of viruses to enter resting lymphocytes could not be completely resolved because the state of activation of the lymphocytes taking up virus particles was not determined because the lymphocyte preparations examined were not depleted of endogenously activated cells. In more recent experiments resting G_o human tonsilar B cells, isolated by size fractionation, were shown to take up measles virus, as these B cells upon subsequent activation with mitogens produced measles antigens on the B cell surface and produced PFUs (McChesney et.al., 1987). Unfortunately, these experiments fail to define the nature of the block in virus infection in unstimulated lymphocytes or the inductive signals necessary to drive resting primary B lymphocytes to a state where they will support a virus infection.

What evidence would suggest that the activation state of the B cell can affect the outcome of a virus infection? EBV can infect and transform normally short-lived resting B lymphocytes into persistently infected

blast cell lines which express some early EBV antigens and are capable of continuous proliferation. Further stimulation (activation) of these cell lines by cross-linking their surface immunoglobulin receptors with anti-immunoglobulin antibody induces cellular changes that result in a lytic EBV infection (Crawford and Ando, 1986). These results, combined with the findings with measles and VSV infections previously discussed, suggest that the eventual outcome of a virus infection is affected by the unique biochemical characteristics found in B cells in different activation states. Moreover, efficient virus replication may require unique host cell factors which may only be present during a particular activation state or available in a particular lymphocyte lineage.

B cell activation and the induction of cellular factors which alter B cell physiology are brought about by engaging two classes of receptors, the receptors for antigen or mitogens on B cells or receptors for lymphokines. Stimulation of the antigen receptors on B cells, either with specific antigen or in a polyclonal fashion using anti-immunoglobulin (anti-Ig), or mitogen receptors, such as lipopolysaccharide (LPS), induce cell enlargement and increased RNA and protein synthesis that support entry into the S phase of the cell cycle.

Differentiation to antibody secretion is supported by LPS activation alone, whereas anti-Ig stimulated B cells require the support of lymphokines. Supplemental signalling is provided by distinct lymphokines that are known to be active during different stages in the B cell activation pathway (O'Garra et.al., 1988). This likely reflects the regulated expression of distinct lymphokine receptors and/or the ability to respond to distinct lymphokines that is concurrent with stages of B lymphocyte differentiation. As with other powerful biological mediators, lymphokines can have opposing effects on B lymphocytes, i.e. the presence of interleukin-4 (IL-4) enhances proliferation and the synthesis of IgG_1 and IgE whereas interferon-gamma suppresses proliferation and the IgG_1 synthesis induced by IL-4 while enhancing IgG_{2a} production in B cells co-stimulated with LPS (Snapper and Paul, 1987). This observation suggests that the unique biochemical changes produced by lymphokines during B cell activation result from the induction of distinct genes, the products of which carry out diverse biochemical reactions. Such changes are likely to affect the B cells ability to support virus infection.

What models are available to assess virus infection in lymphocytes which will permit a critical

evaluation of the impact of cellular activation on this process? Previously, many investigators have used human and murine lymphoblastoid cell lines as stable models of lymphocytes in different lineages or differentiation states for study. Non-permissive cell lines were used as models for non-permissive primary cells in order to investigate the nature of restrictions on virus infection in lymphoid cells. Measles virus was shown to replicate in eight human B and two T cell lines with equivalent efficiency (Joseph et.al., 1975). In contrast, replication of VSV, another negative-strand RNA virus, in a panel of human B lymphoblastoid lines (two of the same lines analyzed in the study of measles infection) was found to be restricted at the level of genomic replication; VSV infection was not restricted in T cell lines (Nowakowski et.al., 1973; Creager et.al., 1981; Johnson and Herman, 1984). Further analysis of human B lymphoblastoid lines revealed that VSV restriction could be correlated with the presence of the EBV genome which when present resulted in persistent rather than lytic VSV infections. EBV negative B cell lines were totally permissive for VSV, and cytopathic effects were seen (Creager et.al., 1982). These results suggest that the transforming event, which allows for proliferation without

further differentiation of these human B cell lines, may markedly influence the ability to support virus infection and as such confound the attempt to correlate lymphocyte differentiation with the process of infection.

Experiments with murine B cell lines which are thought to closely approximate different stages in the B cell activation/differentiation pathway similarly yielded equivocal results. Experiments using a pre-B cell, an immature B cell, as well as mature B cell lines showed that all these cell lines were equally effective as targets for VSV infection (Rup and Scott, 1987). I have also shown that murine cell lines representing different stages in B cell differentiation support VSV, as assessed by infectious center formation and PFU synthesis (Table 1). The pre-B cell line (70Z/3) and the B cell line (BCL1), which represents an immature B cell that can be further activated to antibody secretion, support VSV infection (>102 infectious centers and >5,000 PFU per 10^3 cells analyzed) as do the myeloma (SP2/0) and hybridoma (36-65) cell lines which have characteristics of end stage plasma cells. Freshly isolated or unstimulated cultured primary B cells do not support VSV infection, assessed by the same assays (Table 1), a result which is in agreement with previous observations on the inability of unselected

populations of resting lymphocytes to support virus infection (reviews: Wheelock and Toy, 1973; Bloom et.al., 1976; McChesney and Oldstone, 1987). However, the requirement for activation can readily be seen, as primary B cells which have been activated for 48 hours with lipopolysaccharide support an increase in both VSV infectious centers and PFU synthesis. A further complication in the use of cell lines for these studies is that lymphokines, which can directly affect virus infection, may be secreted at high levels and in different combinations by both B and T cell lines. Therefore, whereas these cell lines may retain some of the characteristics and response capabilities of B cells in different stages of activation/differentiation, all B cell lines tested support VSV, thus precluding their use as critical models to assess activation induced changes in cell function which effect the support of a virus infection.

The studies presented here directly address how the process of lymphocyte activation in primary B lymphocytes alters the cellular response to virus infection. I use as a model system populations of primary resting murine splenic B cells in the G_o phase of the cell cycle which were enriched by depletion of T cells with

antibody and complement followed by counter-current centrifugal elutriation so as to isolate small resting B cells free of other cell types. B cells so isolated may be stimulated to relatively physiologically homogeneous stages in their activation/differentiation pathway (Figure 1) in an inducer dependent fashion. Lymphokine exposure causes B cell blastogenesis with an increase in RNA and protein synthesis, but does not cause proliferation. The lymphokines to be used in activation studies were prepared as cell culture supernatants (SN) isolated following Con A activation of freshly isolated heterogeneous splenic T cells or T cell clones. SNs so obtained contain different populations of lymphokines and all support transit to the G_1 phase of the cell cycle (O'Garra et.al., 1988; Paul, 1089). In B cells cultured for 48 hours with various lymphokine mixtures there was an average 3-4 fold increase in protein synthesis, directly monitored by incorporation of ^{35}S-methionine, over unstimulated but cultured cells (Table 2). B cell entry into the S phase of the cell cycle can be induced by activation with anti-immunoglobulin (anti-Ig)(DeFranco, 1987). This stimulation causes a further increase in protein synthesis on a per cell basis (Table 2). Activation with anti-Ig and lymphokines or lipopolysaccharide (LPS) represent the

most rigorous B cell stimulation regimens, and, while both support B cell differentiation to antibody secretion (Parker et.al., 1980; Howard et.al., 1982), they use different cellular signalling pathways. Anti-Ig signals through B cell antigen receptors in a polyclonal fashion activating the phosphoinositide/protein kinase C pathway (DeFranco, 1987), and LPS binds to another receptor on the B cell and activates an uncharacterized G protein signal transducing pathway (Lei and Morrison, 1988; Bright et.al., 1990; Chen et.al., 1990). B lymphocyte populations, activated in culture with the appropriate inducers for 24 or 48 hours to different stages in their differentiation pathway were assessed for their ability to support virus infection.

The viruses chosen for this study are vesicular stomatitis virus (VSV), a rhabdovirus, and Newcastle disease virus (NDV), a paramyxovirus (Figure 2). Both are enveloped negative-stranded RNA viruses which undergo similar replication cycles in the cell cytoplasm after being taken up by different routes. VSV is endocytosed and the nucleocapsid subsequently released into the cell cytoplasm when acidification of the endosome causes VSV-G protein mediated fusion of virus membrane with the endocytotic vesicle membrane. The NDV nucleocapsid is

released directly into the cell cytoplasm by fusion of the viral membrane with the cell membrane mediated by the NDV-F glycoprotein at neutral pH. Replication involves an initial round of primary transcription of viral mRNA's, 5 for VSV and 6 for NDV, carried out by the RNA dependent RNA polymerase (encoded by the L gene plus NS or NP) associated with the nucleocapsid and packaged in the virion. Primary translation of viral proteins uses the host cell translation machinery and is followed by an amplification of infection through replication of positive sense genomic RNA molecules which function as templates for negative sense genomic RNA synthesis. Assembly and maturation of the virus particles occurs at the host cell membrane (reviews Kingsbury, 1990; Wagner, 1990).

As these viruses encode few proteins, host cell factors not yet completely defined play a role in the process of infection (Emerson, 1985; Kingsbury, 1990). Restrictions on virus infection can be envisioned to occur at a number of stages in the virus replication cycle and could result from the absence of critical host factors or the presence of specific inhibitors.

In this dissertation VSV and NDV are used as probes to analyze the ability of B cells in different activation states to support infection. Chapter 1

determines the B cell activation requirements necessary to support VSV. In chapter 2 the molecular basis of VSV restriction in non-supportive lymphokine stimulated B cell populations is analyzed. These studies identify translational control as one level of restriction. This chapter also describes the modulating effect of lymphokines on virus expression in B cells by showing that lymphokine populations can both enhance and inhibit virus replication, depending on the activation pathway used for B cell induction. Chapter 3 examines the B cell population which is partially supportive of VSV and begins to examine additional levels of restriction on VSV infection. Chapter 4 examines B cell support of NDV and demonstrates that B lymphocyte activation requirements established for one virus are not universal, even for closely related virus families. Chapter 5 presents the overall conclusions and suggested future directions.

Table and Figure legends

Table 1

Analysis of B cell lines and primary B cells for support of VSV infection. Cell lines were infected at a m.o.i. of 10, treated with anti-VSV serum after one hour, and serial dilutions plated onto chick embryo fibroblasts monolayers for infectious center formation or samples were placed in tissue culture for 24 hours and the culture SN collected for PFU assay. Primary elutriated splenic B cells were tested for support of VSV on the day of isolation or were cultured with the indicated stimulus for 48 hours prior to infection.

a infectious centers per 10^3 cells plated, mean of three serial dilutions

b PFU per 10^3 cells cultured, mean of two serial dilutions

c some clumping of cells, counts may be low

d B cells cultured for 48 hours

Table 2

Small elutriated B cells were cultured for 48 hours with the indicated stimulus prior to assay. Cells were labeled for 2 hours with ^{35}S-methionine, lysed, and samples assayed for incorporation by TCA precipitation.

a mean of duplicate determinations

TABLE 1

ANALYSIS OF SUPPORT OF VSV INFECTION BY B CELL LINES REPRESENTING DIFFERENT DIFFERENTIATION STATES

CELL LINE	STAGE	INFECTIOUS CENTERS [a]	PFU [b]
70Z/3	pre B	314	10,000
BCL1	immature B	102 [c]	5,400
Sp2/0	myeloma	297	37,000
36-65	hybridoma	353	21,000
PRIMARY B CELLS			
unstimulated		1	0
unstimulated/cultured [d]		3	2
LPS activated [d]		161	5,960

TABLE 2

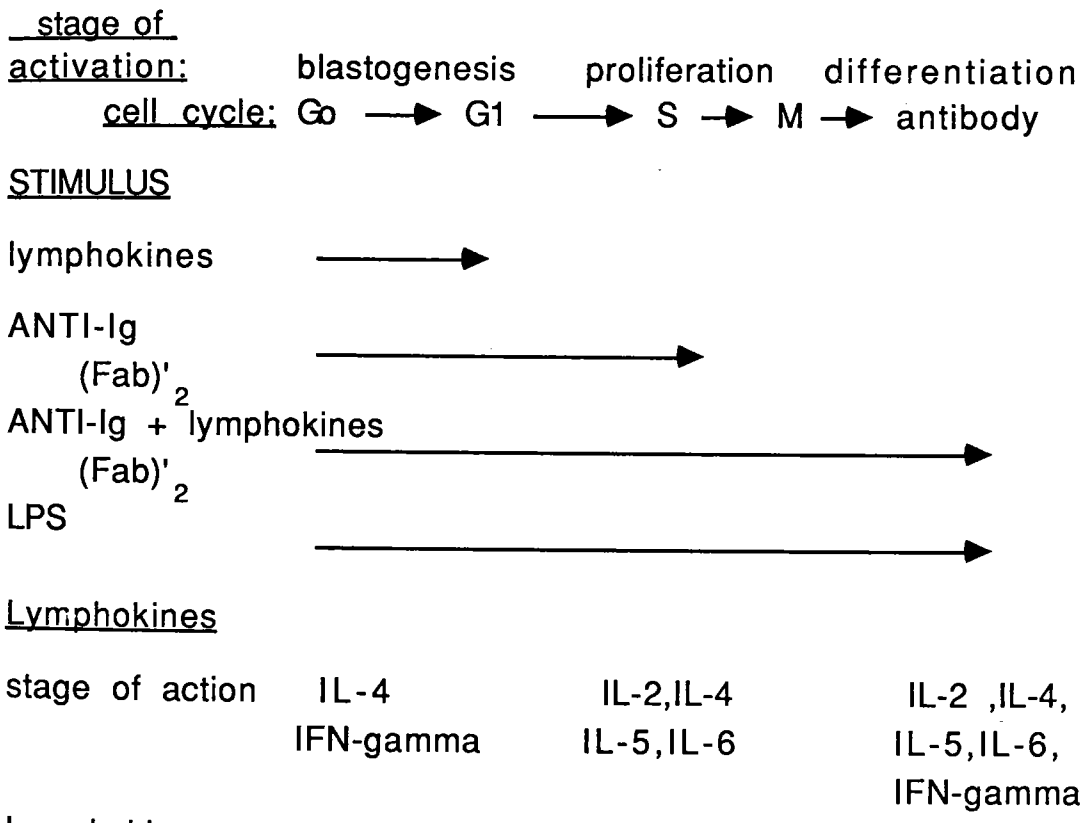
INCREASED PROTEIN SYNTHESIS OBSERVED WITH CHANGES IN THE ACTIVATION STATE

STIMULUS	CPM/10^5 cells[a]	factor of increase
NONE	4,541	1X
Splenic SN	14,677	3X
Th1 SN	12,823	3X
Th2 SN	19,253	4X
ANTI-Ig	35,606	8X
ANTI-Ig + Sp SN	46,594	10X
ANTI-Ig + Th1 SN	48,231	10X
ANTI-Ig + Th2 SN	62,196	14X
LPS	114,477	25X

FIGURE 1

STAGES OF B CELL ACTIVATION AND DIFFERENTIATION AFFECTED BY STIMULATION WITH THE INDUCERS USED IN THIS STUDY

B CELL ACTIVATION



Lymphokine source:

defined lymphokines present in SNs prepared from the indicated cell source

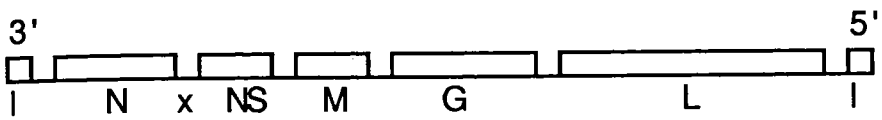
CD 4+, Th_1 (D1.6 SN): IL-2, IL-3, gamma-IFN, lymphotoxin, GM-CSF

CD4+, Th_2 (CDC35 SN): IL-3, IL-4, IL-5, IL-6, lymphotoxin, GM-CSF

polyclonal splenic SN: above lymphokines except low levels of IL-4

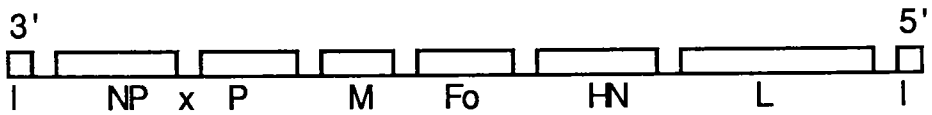
FIGURE 2

VESICULAR STOMATITIS VIRUS



I - leader sequences, 50 NT
N - nucleocapsid, 1326 NT
NS- nucleocapsid associated, 815 NT
M - matrix, inner membrane, 813 NT
G - glycoprotein, spikes, 1665 NT
L - RNA dependent RNA polymerase, 6500 NT
x - intergenic spaces, 2-4 NT

NEWCASTLE DISEASE VIRUS



I - leader sequence, 55 NT
NP- nucleocapsid, 1746 NT
P - nucleocapsid, 1451 NT
M - matrix, 1241 NT
Fo - fusion, 1792 NT
HN- hemagglutinin-neuraminidase, 2031 NT
L - RNA dependent RNA polymerase, 8704 NT
x - intergenic spaces, 0-31 NT

MATERIALS AND METHODS

Mice: CBA/NJ X A.By/J female or A.By/J X 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, ME.

Virus stocks: Vesicular stomatitis virus, VSV, strain Salt Lake City, was obtained from Dr. T. Morrison (Univ. Mass. Med. School) and stocks were prepared in CHO cells and purified as previously described (Chatis and Morrison, 1981). Newcastle disease virus, NDV, strain Australian Victoria, was obtained from Dr. R. Iorio (Univ. Mass. Med. School) and stocks were prepared as described (Hightower and Bratt, 1975).

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 (Mishell and Shiigi, 1980). Pooled spleen cell suspensions were washed twice by centrifugation through modified Hank's Balanced Salt Solution (BSS) supplemented with 1.5% vol/vol fetal calf serum (BSS-FCS) and enriched for B cells by complement mediated depletion

of T cells, i.e. cells were exposed to anti-Thy 1.2 and anti-Ly 2.2 monoclonal antibodies for 1 hour on ice, washed and treated for 1 hour at 37°C with a 1:10 dilution of rabbit complement (Pelfreeze, Rodgers, Ark.) or guinea pig complement (Rockland, Gilbertsville, Pa.) pre-adsorbed on mouse splenocytes.

Cell Separation: Splenocytes enriched for B cells were resuspended to 50 X 10^6/ml in BSS-FCS supplemented with 125 ug/ml DNase I (Sigma, St. Louis, Mo.) 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, Palo Alto, CA.) as previously described (Biron and Welsh, 1982). Cells eluting at a flow rate of 20 ml/min with a symmetrical peak and a mean cell volume of 120 um^3 were collected and defined as the small resting fraction (Tony et.al., 1985)(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 Coulter Channelyzer (Coulter Electronics, Inc., Hialeah, FL.). 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 media (CM): RPMI 1640 supplemented with 10% fetal calf serum, 2mM L-glutamine, 100 units of penicillin, 100 ug of streptomycin, 10 ug of gentimycin, MEM non-essential amino acids per ml and 5 X 10^{-5} M 2-mercaptoethanol. Cells were cultured with various lymphokines and mitogens in 60 mm tissue culture dishes, 5 ml/dish, at a cell concentration of 5 X 10^6/ml, at 37°C in a 5% CO_2 in 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, washed, and triplicate samples of 5 X 10^5/200 ul of viable cells were placed into 96 well microtiter trays with 1 uCi/well of ^{3}H-thymidine (NET-027, 6.7Ci/mM, New England Nuclear). Plates were incubated at 37°C for 6 hour, harvested using a PHD cell harvester (Cambridge Technology Inc., Cambridge, Ma.), and thymidine incorporation assessed by scintillation counting.

Mitogens and Lymphokines: The B cell mitogen, lipopolysaccharide (LPS W, E.coli 055:B5. Difco Laboratories) was used at a final concentration of 50 ug/ml of culture medium. Rabbit anti-mouse F(ab)'$_2$

antiserum (anti-Ig) was obtained from rabbits hyperimmunized with $F(ab)'_2$ fragments of monoclonal antibody UPC10 (kappa,gamma 1). Anti-$F(ab)'_2$ antibodies were affinity purified from hyperimmune serum by adsorption and acid elution from an immunoadsorbent of normal mouse Ig 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 10-25 ug/ml. Lymphokine containing supernatants (SN) were prepared from cloned lines of distinct T helper cell subpopulations; Th_1 - clone D1.6 (Kurt-Jones et.al., 1987); Th_2 - clones CDC25 or CDC35 (Tony et.al., 1985) generously provided by Dr. D. Parker (Univ.Mass.Med.School) by induction for 24 hours with 4 ug/ml of Concanavalin A (Con A, Sigma) 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 ug/ml Con A for 24 hours. Con A was removed from all SNs by passage over a methyl-alpha-D-mannopyranoside sepharose column. All SN were used at a 50% concentration in cultures supplemented with methyl alpha-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)(Marshak-Rothstein et.al., 1979), anti-Ly 2.2 (ATCC# TIB 150), anti-B220 (14.8, (Kincade et.al., 1981) and RA3.3A1 (Coffman and Weissman, 1981)), anti-Ia^k (14.4.4S,(Ozato et.al., 1980), 10.2.16, ATCC# TIB 93; and 11.5.2, ATCC# TIB 94), anti-B cell (J11D, (Bruce et.al., 1981)), anti-rat kappa (Mar 18.5, ATCC# TIB 216). The pre B cell line 70Z/3 (ATCC# TIB 158) and the immature B cell line BCL1 (ATCC# TIB 197) were used as target cells for VSV. Also the B cell hybridoma fusion partner Sp2/0 (ATCC# CRL 1581) or the antibody secreting hybridoma, 36-65 (Marshak-Rothstein et.al., 1980) 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, supplied by Dr. T. Morrison, or rabbit anti-NDV serum, supplied by Dr. R. Iorio, were used to neutralize virus. Monoclonal antibody HN 2b against the NDV hemagglutinin-neuraminidase protein was provided by Dr. R. Iorio.

Assays for Virus Infection: B cells, usually 5 x 10^6 cells cultured for various times under various activation conditions, were infected with VSV at a multiplicity of infection (m.o.i.) of 10 PFU/cell for 1

hour 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 mins. at 37°C cells were washed twice, resuspended to the original volume, and counted. Samples were also taken for an overnight culture to be used in an infectious center assay for determining virus production, and for ^{35}S-methionine labeling of viral and host proteins.

The number of infected cells was determined using an infectious center assay. Briefly, duplicate 0.2 ml aliquots from samples of serially diluted cells were plated onto 60 mm tissue culture dishes of 2° chick embryo fibroblast monolayers. Cell samples were plated in a 1:1 mix of CM containing cells and overlay media (MEM with 2.5% serum, penicillin, streptomycin, gentimycin, tryptose phosphate broth, $CaCl_2$, 0.8% agarose (Seaplaque, FMC biochemicals), and 0.2% agar (Bacto-agar, Difco)). After 1 hour the monolayers were overlayed with an additional 5 ml of overlay media. Virus plaques were counted at 24 and 48 hours.

Virus production (PFU) was assayed 24 hours after infection by incubating 5 X 10^5 infected cells in 1 ml of CM overnight in 24 well tissue culture dishes. SN were collected and infectious virus was titrated on 2° chick

embryo fibroblast monolayers. To examine the effect of trypsin on NDV plaque production, monolayers of 2^{o} chick embryo cells were washed with phosphate buffered saline prior to addition of serially diluted SN and overlayed with avirulent media (Media 199 with penicillin, streptomycin, gentimycin, tryptose phosphate broth, bicarbonate, 10 ug/ml trypsin, 0.4% agarose, and 0.1% agar).

In some experiments small B cells were preinfected with VSV at a m.o.i. of 10 for 2 hrs. at $37^{o}C$, washed, and excess virus neutralized before activation in culture. After 24 or 48 hours in culture these preinfected cells were washed and excess virus was again neutralized before analyzing these cells for infectious center formation and virus production.

Viral protein synthesis was examined by continuous labeling with ^{35}S-methionine for 2 hours of cells infected 8-10 hours previously. Briefly, cells were washed twice in CM made with methionine free RPMI 1640, resuspended at 10 x 10^{6}/ml with 50uCi of ^{35}S-methionine (Amersham #SJ-1515, 1250 Ci/mM specific activity, Amersham Corp., Arlington Heights, IL.) and pulsed for 2 hours at $37^{o}C$. Thirty min. prior to cell lysis, iodoacetamide in PBS (Sigma, St.Louis, Mo.) was added to a final concentration of 7-10

mM. Cells were spun down, SN removed, and cell pellets lysed with lysing buffer (0.5% NP-40, 0.5% deoxycholate, 50 mm Tris, pH 8.0) with phenylmethylsulfonyl fluoride (final concentration 2 mM). Cell lysates were microfuged and the SN were analysed on a 10% SDS acrylamide gel with fluorography.

Immunoprecipitation of viral proteins was carried out by adding protein A-sepharose 4B beads (Sigma, St. Louis, Mo.), previously loaded with rabbit anti-VSV or anti-NDV antibodies for 2 hrs at R.T. and then washed with 0.5% NP-40 in 50mm Tris pH 8.0, to cell lysates or in virto translation products. Samples were incubated for 2 hrs. at R.T., washed 4 times with 0.5% NP-40, 400mm NaCl in 50mm Tris pH 8.0, and radiolabelled proteins were eluted by boiling in SDS gel sample buffer for 5 mins. and electrophoresed on a 10% SDS acrylamide gel.

RNA extraction and Northern analysis: RNA was extracted from infected B cell populations by the acid phenol extraction method (Chomczynski and Sacchi, 1987). Briefly, cell pellets were dissolved in solution D (4M guanidinium thiocyanate, 25mM sodium citrate pH 7, 0.5% sarcosyl, 0.1M 2-mercactoethanol, 100ul/ 10^6 cells). 2M sodium acetate pH 4.0 (10ul/ 10^6 cells), phenol (water saturated, 100ul/ 10^6 cells), and chloroform/isoamyl

alcolol (49:1, 20ul/ 10^6 cells) were sequentially added to the lysates, mixed after each addition with a final vortex of >10 seconds and placed on ice for 15 mins. Preparations were spun at 9,000 rpm for 20 mins at 4°C, upper phases were re-extracted with phenol /chloroform:isoamyl alcohol twice, after a final spin the upper phase was removed and solution D (15ul/ 10^6 cells) was added. One volume of cold isopropanol was added, and RNA was precipitated for at least 1 hour at -20°C. The RNA pellet was microfuged (4°C for 20 mins.), washed with 70% ethanol and dried. Concentrations were determined by optical density after dissolving in sterile distilled water.

RNA was analyzed by Northern blotting. Total RNA was separated on a 1% formaldehyde-agarose gel (Maniatis, 1982) run at 24 volts for 16-20 hours and transfered to Zetabind (CUNO, Inc., Meriden, Ct.) by capillary elution with 10X SSC buffer (Maniatis, 1982) for greater than 20 hours. After transfer, the filter was washed in 2X SSC, baked in vacuum oven at 80° C for 2 hours, washed in 1% SDS, 0.1X SSC at 65° C for 1 hour, and pre-hybridized at 42° C overnight in 50% deionized formamide, 1X Denhardts (Maniatis, 1982), 1% SDS, 5X SSC, 10mM EDTA, 50mM $NaPO_4$ pH 6.7, 0.2 mg/ml yeast tRNA, 0.2 mg/ml denatured salmon

sperm DNA, and 8.2% H_2O.

Plasmid and probe preparation: Plasmids containing complete cDNA sequences for the VSV G (Rose and Bergman, 1982), M (Rose and Gallione, 1981), N (Sprague et.al., 1983), and NS (Gallione et.al., 1981) were obtained from Dr. John K. Rose. A plasmid encoding the VSV L gene was obtained from Dr. Manfred Schubert (Schubert et.al., 1985). These plasmids were used to transform E.coli (strain DHfr alpha) and prepared by standard techniques (Maniatis, 1982). Inserts were prepared by digesting the plasmids with appropriate restriction enzymes and purified from agarose gel using the gene clean system (BIO 101 Inc., La Jolla, CA.). Probes were labeled using the random primer labeling procedure (Feinberg and Vogelstein, 1983): 1 ul of DNA (25 ng/ul) was added to 8 ul of sterile double distilled H_2O, and denatured by heating to 95-100^oC for 3 mins., and iced. After cooling 2 ul $dNTP_6$-10X pol buffer [$pd(N)_6$ (Pharmacia, 135 ug/ml), pol buffer (70mM Tris pH 7.4, 70 mM $MgCl_2$, 0.5 M NaCl)], 1 ul 0.5 mM dC^o (dTTP, cATP, dGTP), 8 ul ^{32}P-dCTP (3000 Ci/mM), and 2-3 units Klenow fragment of DNA polymerase were added. This mixture was incubated 30 mins. at 37^oC, and unincorporated nucleotides were removed by passage over a G-50 sephadex spin column.

The preparation was added to hybridization buffer (same as pre-hybridization), boiled 10 mins., and immediately added to filter which was hybridized overnight at 42°C. The filter was rinsed in 2X SSC, 0.1% SDS for 5 mins. at R.T., washed twice with 0.1X SSC, 0.1% SDS for 45 mins. at 50°C prior to exposure to film at -70°C with developing screens.

In Vitro translation: Translation reactions were carried out in a wheat germ system according to described methods (Erickson and Blobel, 1983). Briefly, 25 ul reactions containing 1 ug of total B cell RNA, 30% (vol/vol) nuclease treated wheat germ extract, and 18.75 uCi ^{35}S-methionine were incubated 90 mins. at 24°C prior to product analysis by SDS gel electrophoresis. Wheat germ extract was supplemented with 25 mM creatine phosphate, 1.8 mM ATP, 0.3 mM GTP, 0.18 mM of each amino acid excluding methionine, 25 mM HEPES (pH 7.8), 64 ug/ml creatine phosphokinase, 250 uM spermidine, 3.2 mM DTT, 0.0016% Nikkol, 7U/25ul RNase inhibitor, 0.9 mM MgOAc, and 20 mM KOAc.

CHAPTER 1

VIRUS-LYMPHOCYTE INTERACTIONS: INDUCTIVE SIGNALS NECESSARY TO RENDER B LYMPHOCYTES SUSCEPTIBLE TO VESICULAR STOMATITIS VIRUS INFECTION

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Material and methods and references have been incorporated into the appropriate sections of the thesis. Tables and figures have been renumbered.

ABSTRACT

We have 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_o phase of the cell cycle, were cultured with stimulators which allow progression through various stages in the activation/ differentiation pathway leading to antibody secretion. We find that VSV expression is dependent on the state of B cell activation, and that three distinct phases can be defined. A non-supportive state, which is defined by the failure to produce infectious centers, viral proteins, or PFUs, is characteristic of freshly isolated small B cells, B cells cultured 48 hours 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_o B cells with anti-immunoglobulin, at doses which allow entry into S phase, rendered them capable of synthesizing viral proteins and increased the number of B cells producing infectious 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/infectious center). Lipopolysaccharide stimulation, acting through another induction pathway, caused the maximum increase in the number of infected B cells and infectious particle production (25 PFU/infectious center).

INTRODUCTION

The alarming increase in virus mediated immunodeficiency disease 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 (Coffman and Weissman, 1981; Cooper et.al.,

1988). B cells stimulated to enter the S phase of the cell cycle lose their susceptibility to EBV because of the downregulation of the CR2 molecule from the cell surface (Casali and Notkins, 1989; Crawford and Ando, 1986; Tovey et.al., 1978).

The necessity to induce critical biosynthetic pathways in the target cell provide 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 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_O) resting phase of the cell cycle; in this state the cells have little cytoplasmic volume (Phillips and Parker, 1984), minimal biosynthetic capability (Howard et.al., 1982; Paul, 1989), and are poor hosts for viral infection (Bloom et.al., 1976; Jimenez et.al., 1971; Olding et.al., 1975). 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 (Bloom et.al., 1976; Edelman and Wheelock, 1966;

McChesney and Oldstone, 1987; Wheelock and Toy, 1973).

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 (DeFranco, 1987) 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_o 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 (DeFranco, 1987; Paul, 1989). The physical changes in activated B cells allows 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 in resting B cell populations exposed to defined activation inducers that were chosen based on their following propertities: (1) Exposure of small resting B cells to anti-Ig plus lymphokines derived from T helper cells or to the mitogen LPS allows full activation causing both proliferation and differentiation to antibody secreting cells (Howard et.al., 1982; Parker et.al., 1980); (2) Incubation with anti-Ig, causes small resting G_o B cells to enter S phase without further differentiation (DeFranco, 1987); and (3) Lymphokines, in particular IL-4, support B cell transit into the G_1 phase of the cell cycle (O'Garra et.al.,1988; Paul, 1989).

We have analyzed changes in B cell susceptibility to infection using Vesicular Stomatitis Virus. VSV is a negative-stranded RNA virus in the Rhabdovirus family. Replication occurs in the cytoplasm of the infected cell and is dependent upon host cell factors (Emerson, 1985; Wheelock and Toy, 1973). We followed the process of infection experimentally in B cell populations by: 1)

using SDS gel electrophoresis of ^{35}S-methionine labelled cell lysates to ascertain viral protein synthesis, 2) the number of infected cells in the population is determined by an infectious center formation, and 3) titering supernatants of infected cultures for the production of infectious virus particles

We find 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.

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 Ig (J. Riggs, personal communication). Table

3, Expt. 1 shows the number of infectious centers/10^3 plated cells exposed to different culture conditions for 48 hours. B cells cultured for 48 hrs. with no additional stimulus produce 39 infectious 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 infectious centers, while B cells which received inductive signals from anti-Ig, which allows transit to S, produced 200 infectious centers. Cells which were exposed to more rigorous stimulation with anti-Ig plus splenic SN or LPS produced more infectious centers, $340/10^3$ and $300/10^3$, respectively. Thus, up to 30% of the cells activated with anti-Ig 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%). Expt. 2 and others not shown yield similar results.

These data also imply that B cells are the target for VSV infection, given that 95% of the starting cell population is sIg^+, and the specificity of B cell induction with anti-Ig and LPS activators. Moreover, because not all B cells are activated to proliferation by LPS or anti-Ig (30 and 60% respectively; Ashman, 1984;

DeFranco et.al., 1982), 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 establishing the target population, the previous experiments 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 infectious center assay ($4/10^3$ plated cells) or virus production (Table 4, Expt. 1). Moreover, B cells maintained in culture for 48 hours without stimulation also showed no increase in number of infectious centers ($3/10^3$ plated cells), thus demonstrating that no inducers are found in culture medium. This small number of infectious 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 3). 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.

Infectious center formation markedly increased in small B cells exposed to B cell activators. In this regard, LPS activation was the most effective, at 48 hours there was a 65 fold increase over the small B cell background to 195 infectious centers per 10^3 cells plated; exposure to anti-Ig with or without suplementation with lymphokines caused 20 to 30 fold increases, respectively.

The most striking change brought about by variation in activation stimulus was in infectious particle production. B cells stimulated with anti-Ig had increased numbers of infectious centers, $90/10^3$ cells plated, and increased PFU produced, $52/10^3$ cells cultured, but the virus yield on a per cell basis was less than 1 PFU/infectious center, which is comparable to that seen in unstimulated B cell cultures. In contrast, anti-Ig plus SN increased PFU formation 200 fold overall, and also increased virus production on a per cell basis to 7 PFU/infectious 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/infectious center, a value close to the level of virus production seen in permissive cell lines. In other experiments B cells were cultured with anti-Ig and SN from

Th_2 T cell clones (Table 4, Expt. 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-Ig plus splenic SN cultures. These data demonstrate that both the number of cells infected and the level of PFU production are effected by the activation stimuli.

The progress of infection was also monitered by examining viral protein synthesis. As expected VSV proteins are readily seen in productively infected B cells stimulated by anti-Ig and SN or LPS (Figure 3, lanes 4 and 8). VSV protein synthesis is also seen in cells stimulated with anti-Ig despite the fact that low numbers of infectious particles were produced (Fig. 3, lane 2). Immunoprecipitation shows that the lack of PFU production in anti-Ig 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 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-Ig and SN. Treatment with anti-B220, anti-Ia, and J11D resulted in a decrease in cell numbers of 37%, 88%, and 78% of cultured cells with a corresponding decrease in infectious 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 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. 4, stimuli 5-9). Yet the data for anti-Ig stimulated cells shows that entry into S is not sufficient for a productive VSV infection. However, B cells cultured with anti-Ig 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_o to G_1 cell cycle transit in small B cells causing increases in B cell volume, RNA synthesis, and protein synthesis (O'Garra et.al., 1988; Snapper et.al., 1988) 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 Con A activated splenic T cells or from T helper cell clones. T helper cell clones represent two distinct T helper subpopulations (T_h1 and T_h2) (27). T_h1 cells secrete IL-2 and gamma-interferon, while T_h2 cells secrete IL-4, IL-5, and IL-6 as their unique lymphokines, whereas both secrete IL-3, lymphotoxin, GM-CSF. Small B cells exposed to these SNs for 48 hours produced no infectious centers, PFUs (Table 5) or viral proteins (fig 3, lane 7, splenic SN). The SNs did induce the expected increase in B cell size (fig. 5) and protein synthesis, indicated by a 10-fold increase in ^{35}S-methionine incorporated into TCA 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 non-permissive 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 to 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 neutralized. Cells so treated were cultured without stimulation, or were exposed to splenic SN, or LPS for 24 and 48 hours. A representative expt. using this approach is shown in Table 6, part 1. At 24 hours there is no significant increase in infectious foci in any population which demonstrates the requirement for B cell activation

for virus expression. By 48 hours there is a marked increase in the number of infectious centers in the LPS stimulated cultures, $122/10^3$ plated cells, as well as PFU production, $1,700/10^3$ cells cultured, which are comparable values to those obtained when B cells are activated with LPS before VSV exposure (Table 6, part 2). In contrast, unstimulated B cells or B cells exposed to lymphokines remain non-permissive. 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.

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 maturation 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 (Bloom et.al., 1970; Bloom et.al., 1976; Kano et.al., 1973; Webb et.al., 1981) 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 counter-current elutriation, and the use of LPS or anti-Ig 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-Ig fails to induce cells capable of supporting viral protein synthesis or infectious virus particle production, thus excluding the possibility that a quiescent contaminating cell population is enriched or activated by lymphokine exposure. We also find 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-Ig and T cell-derived lymphokines.

Virus infection in activated B cells is efficient. In B cell populations cultured with LPS or anti-Ig and lymphokines the relative proportion of susceptible cells,

as monitered by infectious center formation, is comparable to the fraction of B cells known to be stimulated to proliferation under the conditions used in these experiments (Ashman, 1984; DeFranco et.al., 1982). Moreover, virus production, as monitored by infectious particle titer, is similar to that found when conventional VSV-susceptible target populations, such as CHO cells, are analyzed.

Activation is clearly necessary to render B cells susceptible to infection, as small resting B cells in the G_o 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 reqires 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_o to G_1 and B cell enlargement (Fig. 4), 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, Abstr., ICN-UCI Int. Conf. on Virol., 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-Ig produced viral proteins and showed a 30-fold increase in infectious centers over background, but failed to produce infectious virus particles at a high rate (less than one per infectious center). This apparent disparity between the number of infectious centers and PFUs detected in SNs 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-Ig stimulated B cells have some characteristics of persistently infected cells which also produce no or low levels of infectious particles. The restriction on infectious particle production in anti-Ig 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-Ig alone was sufficient to render B cells capable of high level infectious particle production.

Activation of B cells with anti-Ig plus lymphokines results in a fully permissive cell population with a 10-fold increase in infectious virus particle production per infectious center over that seen with B cells treated with anti-Ig alone. These results are consistent with a requirement for at least two inductive signals to render B cells supportive of VSV production. Anti-Ig mediates receptor crosslinking 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 Con A activated spleen cell SNs 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 (Paul, 1989) has minimal

anti-viral activity on the B cells activated with anti-Ig. It has been reported that gamma-interferon acts as a growth factor rather than an anti-viral factor for activated T cells (Landolfo et.al., 1988). Alternatively, our finding that SN prepared from Th_2 clones, which secrete no gamma-interferon, facilitates VSV production in anti-Ig treated B cells as well as but no better than spleen cell SN (Table 4) may suggest that the response to "enchancing" 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 (O'Garra et.al., 1988).

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 3) and 3-fold over that seen in anti-Ig plus lymphokine treated cultures. Although different pathways of activation are utilized by these two classes of activators (DeFranco, 1987), 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, K. A. Gravel, and R. T. Woodland, 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-Ig. 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-Ig may represent stable and distinct B cell subpopulations (Ashman, 1984; Flahart and Lawton, 1987) which differ in the lymphokines to which they respond, or respond differently to the same lymphokines. Alternatively, LPS and anti-Ig 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 B cell's potential to respond to the same collection of lymphokines. Additional studies are in progress to distinguish amongst these possibilities.

Restrictions on VSV expression in transformed

lymphoblastoid cell lines have been reported previously (Creager et.al., 1981; Creager et.al., 1982; Nowakowski et.al., 1973a; Nowakowski et.al., 1978b; Weck and Wagner, 1978). However, it is not always possible to determine how the process of transformation may have altered the course of virus expression as it would have occured in normal cells at equivalent stages of differentiation or activation. In this report, we describe a system to correlate the biochemical changes occuring 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 acting in concert with other stimulators, which may not have been detected by previous methods.

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Table and Figure legends

Table 3

a B cell enriched splenocyte populations were cultured with the indicated stimuli for 48 hours before virus infection.

b Mean of duplicate determinations 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 infectious centers per 10^3 cells plated; in experiment 2, CHO cells gave 210 infectious centers per 10^3 cells plated.

c ND, Not done.

Table 4

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

b Mean of duplicate derterminations averaged over three

serial dilutions. ND, Not done.

c Number of infectious particles per infectious center (IC).

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

Table 5

a Elutriated small B cells were cultured with the indicated stimulus for 48 hours before virus infection.

b Mean of duplicated determinations averaged over three serial dilutions.

c Number of infectious particles per infectious center (IC).

d Cells cultured for 48 hours without additions.

Table 6

a In expt. 1, small B cells were infected on the day of isolation and then cultured for 24 or 48 hours with the indicated stimulus before assay. In expt. 2, another sample from the same population of small B cells was cultured for 48 hours with the indicated stimulus prior to VSV infection.

b Mean of duplicate determinations averaged over two serial dilutions.

c Number of infectious particles per infectious center.

Figure 3

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-Ig (lanes 1 and 2), anti-Ig and 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 proteins (N/NS), and membrane protein (M).

Figure 4

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

Figure 5

Cell volume profiles of elutriated B cell populations. Shown are results for freshly isolated small

B cells (_____) or populations cultured for 48 hours unstimulated (····) or stimulated with splenic SN (_ · _), Th_1 SN (D1.6) (_ _), or Th_2 SN (CDC 35) (_ ·· _).

TABLE 3

B CELL ENRICHED SPLEEN POPULATIONS
ARE TARGETS FOR VSV INFECTION

STIMULUS[b]	INFECTIOUS CENTERS[a] Expt. 1	Expt. 2
NONE	39 (11)	ND[c]
Sp SN	90 (26)	45 (21)
ANTI-Ig	200 (58)	208 (99)
ANTI-Ig + SN	340 (98)	277 (131)
LPS	300 (87)	165 (78)

TABLE 4

ACTIVATION OF SMALL RESTING B CELLS
TO SUPPORT VSV INFECTION

STIMULUS [a]	INFECTIOUS CENTERS [b]	PFU [c]	PFU/I.C. [d]
Expt. 1			
NONE [e]	4	1	<1
NONE (cultured)	3	2	<1
ANTI-Ig	90	52	<1
ANTI-Ig + Sp SN	62	450	7
LPS	195	5,300	27
Expt. 2			
ANTI-Ig	ND	51	
ANTI-Ig + Th2 SN	ND	395	
LPS	ND	2,050	

TABLE 5

INABILITY OF SMALL B CELLS STIMULATED WITH LYMPHOKINE-RICH SN TO SUPPORT VSV INFECTION

STIMULUS [a]	INFECTIOUS CENTERS [b]	PFU [c]	PFU/I.C [d]
NONE [e]	1	4	4
Splenic SN	3	8	3
Th1 SN (D1.6)	4	2	<1
Th2 SN (CDC 35)	6	12	2
ANTI-Ig	110	58	<1
LPS	330	8,560	26

TABLE 6

INFECTIOUS CENTER FORMATION AND VIRUS PRODUCTION IN SMALL RESTING B CELLS FOLLOWING VSV EXPOSURE AND IN VITRO CULTURE

STIMULUS [a]	INFECTIOUS CENTERS [b]		PFU [c]	PFU/I.C. [d]
	24 hr.	48 hr.		
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

FIGURE 3

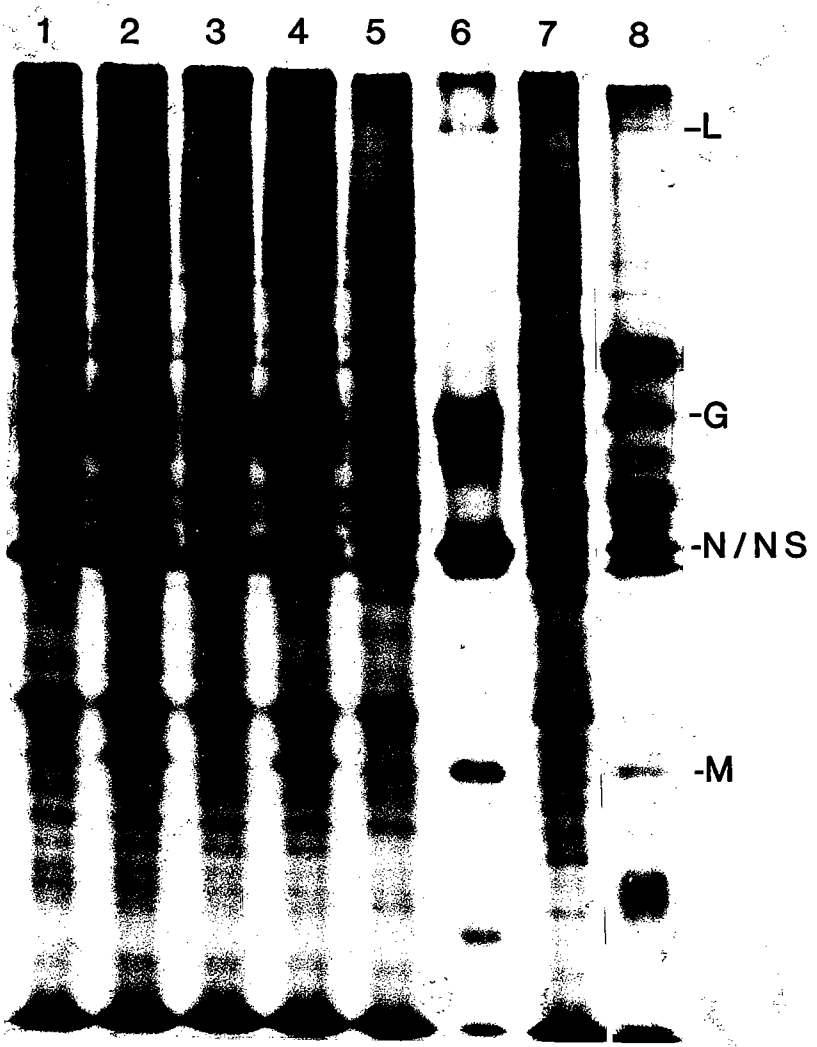


FIGURE 4

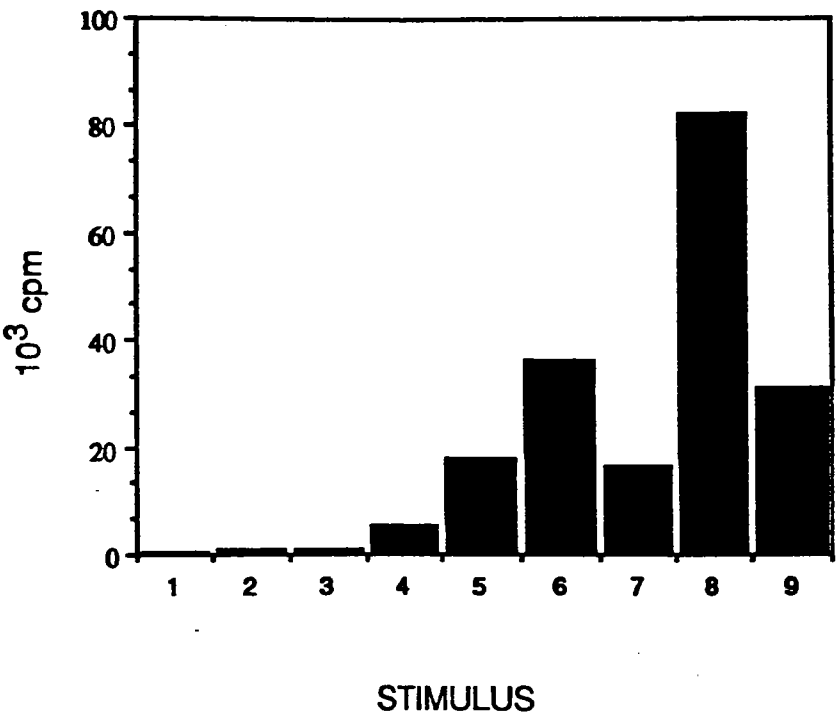
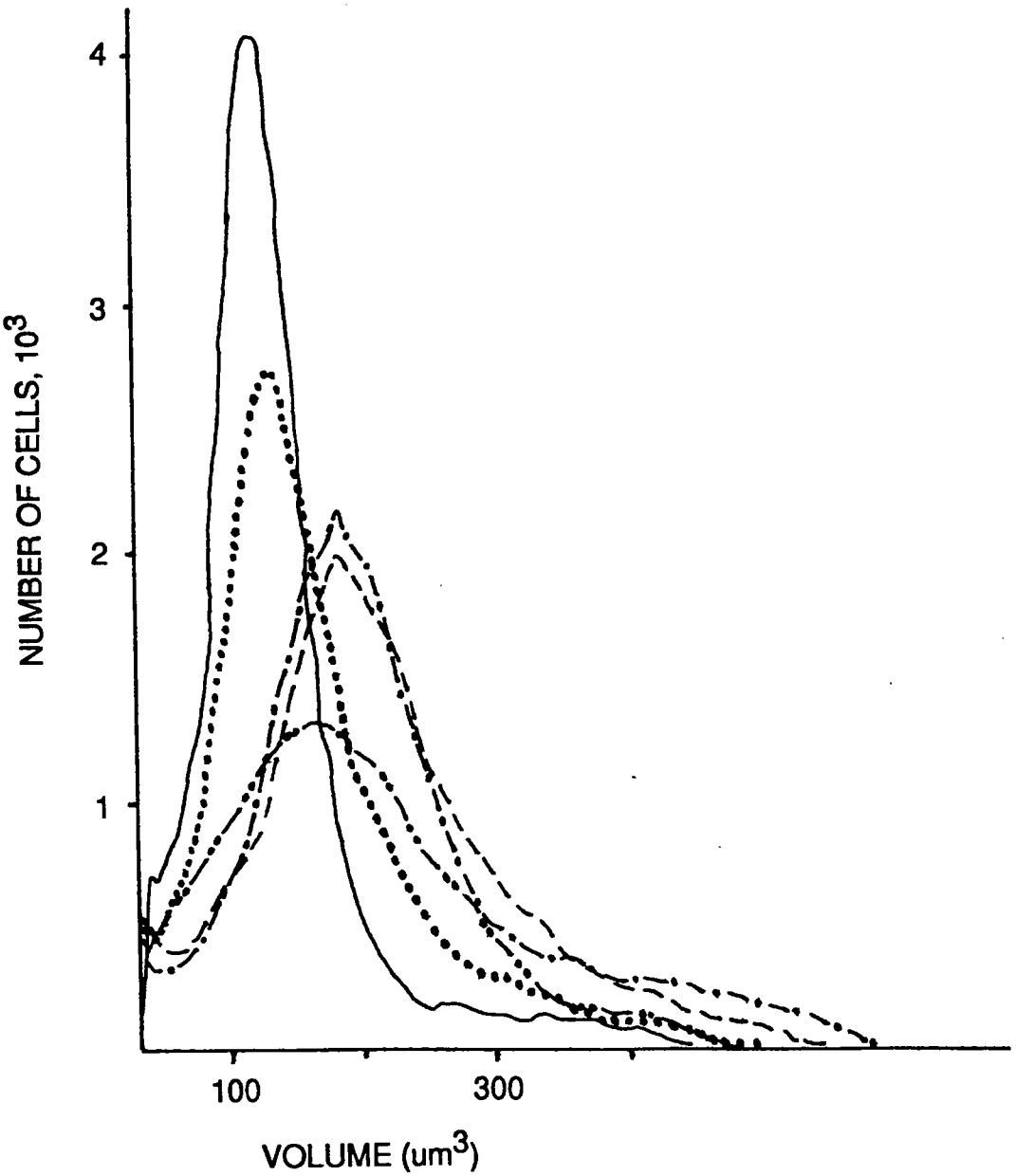


FIGURE 5



CHAPTER 2

Lymphokine effects on VSV non-supportive and supportive B cell populations

INTRODUCTION

Lymphokines are powerful regulators of B cell function. The requirement of B cells for extraneous factors to facilitate antibody secretion, proliferation, and isotype switching has been used to define the function of particular lymphokines and has provided a model to study the molecular basis of lymphokine action. For example, B cells activated with LPS when co-cultured with recombinant IL-4 produce IgG_1 and IgE, whereas B cells activated with LPS plus IL-4 and IL-5 preferentially synthesize IgA (Coffman et.al., 1988; Snapper et.al., 1988). This situation is also rendered more complex by the finding that a particular lymphokine not only affects immunoglobulin isotype, but the amount of lymphokine also influences antibody synthesis, e.g. *in vitro* synthesis of IgE requires 10-100 times more IL-4 than that required for IgG_1 synthesis (Snapper et.al., 1988). Lymphokines can also have opposing effects. Interferon-gamma suppresses B cell proliferation that is normally supported by IL-4 and inhibits the IL-4 mediated synthesis of IgG_1 and IgE while

enhancing IgG_{2a} synthesis. Moreover, lymphokine action is redundant in that different defined lymphokines may have similar effects or provide similar support for B cells. This is demonstrated by the ability of either IL-4 or IL-5 to support B cell proliferation and differentiation to antibody secretion (Figure 1, introduction; O'Garra et.al., 1988; Paul, 1989). In summary, lymphokine action on B cells is intricate, including interaction among lymphokines themselves as well as with other types of B cell stimulators, such as antigen or polyclonal activators like LPS and anti-Ig.

The action of lymphokines, like that of other biological mediators, is dependent on the expression of highly specific receptors which may be either constitutively expressed or induced as a result of B cell activation from the resting G_o state (see Figure 1, introduction; review: O'Garra et.al., 1988). Accordingly, lymphokines can have direct effects on resting G_o B cells acting through constitutively expressed lymphokine receptors. For example, IL-4 acting through the IL-4 receptor induces a resting G_o B cell to undergo a number of biochemical changes including increased cell size, RNA and protein synthesis resulting in the induction of new lymphokine receptors, such as the high affinity chain of

the IL-2 receptor (Loughnan and Nossal, 1989). Because the process of activation via mitogen or lymphokine signalling may lead to the induction of new lymphokine receptors, a broader range of responses is possible in B cells that have been previously activated or co-stimulated with other inducers. These considerations suggest that the specific lymphokines, the concentration of lymphokines, and the array of receptors expressed at a particular stage of B cell activation may influence B cell functions. I have used the assay of B cell support of VSV infection as a new model system for assaying biochemical capabilities of B cells exposed to lymphokines. The focus of the studies presented here is to determine how lymphokines affect the ability of B cells in different activation stages (or exposed to different activation signals) to support VSV infection.

I have previously shown that the ability of B lymphocytes to support VSV infection is variable and dependent on the activation state of the host B cell (Schmidt and Woodland, 1990). Unstimulated B cells in the G_o phase of the cell cycle and lymphokine stimulated B cells in the G_1 phase of the cell cycle infected with VSV demonstrate the same non-supportive characteristics, including lack of viral protein synthesis, infectious

center formation, or PFU production, this despite the ability of virus to enter these cells. The restriction of VSV infection in these non-supportive B cell populations could be at multiple levels, such as 1) release of the viral nucleocapsid from the endocytotic vesicle, 2) primary transcription of viral mRNAs, or 3) translation of the viral mRNAs.

Studies presented here investigate the transcription of specific viral mRNAs in unstimulated and lymphokine stimulated populations of B cells to begin to determine the nature of the block on VSV infection. A lack of viral transcription would suggest a problem with release of the nucleocapsid from the endosome or host cell inhibition of viral transcription. Synthesis of viral mRNAs would directly demonstrate that VSV nucleocapsids are released into the cytoplasm of the B cells and suggest that synthesis of non-functional mRNAs or host cell inhibition of infection at the level of translation accounts for the lack of VSV protein synthesis seen in these B cell populations. The latter possibilities can be distinguished by testing the translatability of the viral mRNAs produced in an <u>in vitro</u> translation system.

This chapter also evaluates another issue about lymphokine action on B cells. It is possible that VSV

infection may be restricted by different mechanisms in unstimulated and lymphokine stimulated non-supportive B cell populations (Table 2, Figure 5). Even though lymphokines stimulate RNA and protein synthesis, the similar level of viral support observed with these populations suggests that metabolic activity per se is not a sufficient signal for "successful" VSV infection. It is possible that lymphokine stimulation directly mediates or induces in these B cells anti-viral effects which mimic the non-supportive state observed in unstimulated B cells. Indeed, the converse seems true, as the addition of these same populations of lymphokines to B cells co-stimulated with anti-Ig produces a marked enhancement of VSV PFU production in these B cells compared to those stimulated with anti-Ig alone (Table 4, 450 vs. 52 PFUs). However, the level of infectious virus production never approaches that found in B cell populations activated with the mitogen LPS (Table 4, 450 vs. 5,300). As complex mixtures of lymphokines are present in these T cell-derived SNs, even those from cloned T cell lines, it is possible that both positive and negative regulators of VSV infection/replication are present. As the ability to repond to lymphokines is dependent on the presence of and the level of expression of receptors, cells with different

receptors will likely respond differently to the same lymphokine mixtures. These considerations suggest that there may also be another level of variability in regards to lymphokine action affecting virus expression, i.e. the ability of B cells to respond to particular lymphokines. Because activation influences lymphokine receptor expression, and because different B cell activation pathways are utilized by anti-Ig and LPS stimulation, it is possible that variation in expression of lymphokine receptors is induced and can subsequently be reflected in the efficiency at supporting a virus infection. A comparison of VSV infection in B cells co-stimulated with either anti-Ig or LPS and lymphokines is also carried out in order to begin to address the possibility that lymphokine effects on virus infection vary depending on the particular activator(s) to which a B cell is exposed.

RESULTS

VSV mRNA transcription in non-supportive B cells

A Northern blot analysis was performed to determine if the block to VSV infection in non-supportive B cell populations was at or before primary transcription of the viral genome. Total cellular RNA was extracted at 9 hours post-infection from B cells cultured for 48 hours

under conditions previously defined as non-supportive (unstimulated or lymphokine stimulated) or supportive (anti-Ig, anti-Ig and lymphokines or LPS stimulated) for VSV infection. In addition, RNA was isolated from the permissive hybridoma cell line 36-65 to serve as a positive control for all experiments. RNA was separated by electrophoresis on denaturing gels, blotted onto nitrocellulose membranes, and probed for primary viral RNA transcripts. Hybridization was carried out with labeled cDNA probes specific for each of the VSV gene products (Figure 6: Panel A, NS mRNA; B, G mRNA; C, M mRNA; D, L mRNA; E, N mRNA; and F, ethidium stain). Hybridization to RNA from VSV infected 36-65 demonstrates that the probes hybridize with RNAs of the appropriate molecular weight for each of the VSV genes analyzed (Figure 6, two lanes labeled 36-65 have 2 and 5ug of total RNA loaded, respectively).

RNAs for each viral gene are readily detected in total cellular RNAs isolated from unstimulated cultured B cells, lymphokine stimulated B cells, anti-Ig and lymphokine stimulated, and LPS activated B cells (Figure 6, A-E). The paucity of viral message found in anti-Ig stimulated B cells is due to underloading of the gel (assessed by ethidium stain, figure 6-F); longer exposure

of the film reveals hybridization with each viral probe (ex. VSV M mRNA, figure 6-C). These results indicate that primary viral transcription occurs in all populations of B cells examined, including those B cell populations in the G_o and G_1 of the cell cycle in which no viral proteins are synthesized (Figure 3, lane 7). Moreover, the presence of viral transcripts in unstimulated B cells also supports the previous conclusion, reached using a different experimental approach, which suggested that VSV enters all of the B cell populations examined (Schmidt and Woodland, 1990).

Culture of resting B cells with lymphokines from helper T cell clones (Th2 SN) also appears to enhance the level of viral transcription over that seen in unstimulated B cell populations without affecting viral protein synthesis. This finding is consistent with the observation that lymphokine mediated activation of B cells from a small resting G_o cell into the G_1 phase of the cell cycle induces transcription and/or translation of a number of endogenous genes, increase in cell size, and the total amount of RNA per cell. For example, resting B cells stimulated with IL-4 increase transcription and expression of class II MHC molecules, CD23 (Fc RII), and the IL-4 receptor (Paul, 1989). While VSV mRNA transcription is

carried out in the cell cytoplasm by virally encoded RNA dependent RNA polymerase and not by the host cell's enzymatic machinery, B cells exposed to lymphokines may upregulate expression of host cell factors which serve to enhance the efficiency of viral transcription. Thus, increases in viral mRNA levels in G_1 B cells may be a direct consequence of lymphokine stimulation of the B cells.

An analysis was carried out in order to more precisely quantitate the effect of lymphokines on viral mRNA transcription amongst B cell populations. The usual strategy for such a quantitation is to determine the level of transcription of selected cellular genes, like actin and glyceraldehyde-3-phosphate dehydrogenase (GADPH), as a background for the comparison for viral transcription levels. However, this approach was complicated by my finding that the expression of these genes is differentially regulated during B cell activation. The Northern analysis in Figure 7 shows that when equivalent amounts of total RNA, determined by optical density and ethidium staining, are analyzed the level of actin mRNA in unstimulated B cells (lane 3) is 2-fold higher than that found in lymphokine stimulated B cell populations (lane 4). In contrast, the GADPH mRNA level in unstimulated B

cells is 2-fold lower than lymphokine stimulated B cells. These differences in internal "standard" RNAs made a straight forward quantitation of viral mRNA difficult. Therefore, viral mRNA transcript levels between unstimulated and lymphokine stimulated B cells populations were compared by Northern blotting using densitometry and three analytical conditions: 1) directly measuring differences in intensity of hybridization when equivalent amounts of total RNA are probed (Table 7A), 2) comparing hybridization intensity between viral mRNA and viral genomic RNA when equivalent amounts of total cellular RNA are probed (this approach assumes that without viral protein synthesis there is no genomic replication (Emerson, 1985) and equal virion uptake, (Table 7B), and 3) determining viral mRNA levels for equal B cell equivalents (Table 7C). When analyzed by these three criteria, viral mRNA transcription was 5 to 10-fold higher in lymphokine stimulated verses unstimulated B cells. However, lymphokine stimulation, while resulting in a marked increase in viral mRNA transcription, is not sufficient for a productive VSV infection. Because primary viral mRNA transcription can occur in B cells without the synthesis of viral proteins, one block to VSV infection is likely at the level of translation in G_o and

in lymphokine activated G_1 B cell populations.

In Vitro translation of VSV mRNAs

The presence of viral mRNAs transcripts without the synthesis of viral proteins demonstrates an inability to translate viral mRNA transcripts. This impairment may be due to the synthesis of defective viral mRNAs (possibly uncapped or with truncated polyadenylation sequences) or result from host cell mediated translational regulation. Both mechanisms are known to modulate viral protein production in other systems. Undermethylation and suboptimal polyadenylation of VSV mRNAs was found to contribute to the aborted VSV infection of Raji cells (Johnson and Herman, 1984), whereas specific host cell restriction on virus mRNA translation has been demonstrated for mouse mammary tumor virus mRNAs (Vaidya et.al., 1983). In this latter case host-imposed restrictions are complex, as translational restriction was associated with three unlinked, independently segregating genetic loci. In our system, host cell restriction on translational efficiency is suggested by the finding that viral mRNAs, of appropriate molecular weights compared to those found in fully supportive B cell populations, are synthesized in both unstimulated and lymphokine-stimulated non-supportive B cell populations (Figure 6). That viral

mRNA transcription levels differed between these B cells without changing the non-supportive phenotype also supports host cell control at translation (Table 7).

The translatability of the viral mRNAs was assessed *in vitro* using a wheat germ translation system. Figure 8 shows the total protein produced by translation of input RNA from a variety of non-supportive and supportive B cell cultures. Total RNA isolated from unstimulated cultured B cells (lane 1) and lymphokine stimulated B cells (lane 2 and 3, 1 and 2ug of RNA added to the translation reaction, respectively) produce, upon translation, bands of ^{35}S-methionine labelled proteins of the appropriate size for viral proteins that co-migrate with proteins produced by the translation of mRNA from fully permissive B cell populations, anti-Ig and SN and LPS stimulated (lanes 4 and 5, respectively) and the B cell hybridoma 36-65 (lane 6). As a standard for viral proteins, labelled viral proteins produced by VSV infection of 36-65 are also included (lane 9). The lower molecular weight of the protein band corresponding to the VSV G protein produced by *in vitro* translation (labeled G_o) is due to the absence of glycosylation in the wheat germ system used.

That these translation products were viral

proteins was confirmed by immunoprecipitation of the translation mixture using rabbit anti-VSV serum (Figure 9). Viral proteins are precipitated from the translation products of unstimulated and lymphokine cultured B cell mRNA (lanes 1 and 2) as well as from the translation products of mRNA isolated from fully permissive B cell populations (lanes 3 and 4).

The amount of G protein found in the in vitro translation products is relatively low in all B cell populations, including products of mRNA from LPS or anti-Ig and SN stimulated cultures, which fully support VSV protein synthesis, suggesting a problem of the efficiency of the translation system rather than the translatibility of the mRNAs. The efficiency may be affected by competition with other mRNAs as well as by the relative abundance of G mRNA in the total RNA pool. The finding that higher molecular weight proteins in general are synthesized with lower efficiency by in vitro translation systems also helps explain this observation.

These data strongly suggest that the lack of viral protein in unstimulated and lymphokine treated B cell populations is not the result of defective viral mRNA's, being produced in these cells, but rather results from a translational block that exists in these B cell

populations. Lymphokines have been shown to upregulate both transcription and translation in B cells, but, this is clearly a specific rather than a generalized effect as VSV protein translation is not induced by lymphokine exposure, despite the fact that viral transcription is enhanced. The data also suggest that lymphokines per se do not induce the translational block as B cells co-stimulated with anti-Ig and lymphokines are fully permissive for VSV.

Stimulation with anti-Ig is known to drive B cells further through the activation/differentiation pathway than exposure to lymphokines alone. This progression is accompanied by a new biosynthetic potential of the B cells which is shown here by the ability of anti-Ig treated B cells to translate viral mRNAs. That the addition of lymphokines to anti-Ig stimulated B cells enhances VSV PFU synthesis over that seen with anti-Ig stimulation alone suggests that lymphokines may complement the activation events supported by other activators. It is also possible that activation increases the ability to respond by inducing receptors on activated cells that were absent on resting cells, thus increasing the response potential of the B cells for lymphokines in the SNs. These differences in response potential in turn affect the B cell ability to

support virus infection. Some of these possibilities will be examined in the next section.

Contrasting effects of lymphokines on VSV expression in supportive B cell populations

Anti-Ig activation, which mobilizes the antigen receptor in a polyclonal fashion on the B cell, induces cell enlargement and entry into S phase of the cell cycle without supporting differentiation of the B cell to antibody secretion. However, the inclusion of T cell derived lymphokines enhances proliferation and promotes differentiation to antibody secretion. This activation protocol has been frequently used as a polyclonal model for the events in thymus dependent clonal activation of antigen specific B cells. It is noteworthy that the multiple signals required for productive antibody expression using this pathway are analogous to the requirements I have established to be necessary to render B cells fully permissive to support a VSV infection. B cell populations cultured with anti-Ig and lymphokines support a productive VSV infection.

LPS activation of small resting B cells also produces a VSV supportive population of cells. LPS utilizes a different cellular pathway for activation in which T cell derived lymphokines are not required for

differentiation to antibody secretion. This is used as a polyclonal model for thymus independent activation of specific B cells.

Although stimulation with LPS or anti-Ig and lymphokines produces antibody secreting cells, it is not known whether all splenic B cells are capable of responding to each of these inductive pathways or whether non-overlapping subpopulations of B cells exist which respond, by antibody secretion, to one but not the other of these activation pathways. Experiments have demonstrated that more than 90% of splenic B cells undergo blastogenesis upon stimulation with either LPS or anti-Ig (DeFranco et.al., 1982; Phillips and Parker, 1984; Sieckmann et.al., 1981; Anderson et.al., 1977), yet only about a third of the stimulated B cells continue to differentiate to antibody secretion (Ashman, 1984; DeFranco et.al., 1982), allowing the possibility that the populations of responding B cells may exist as non-overlapping subpopulations. That B cells stimulated by these thymus dependent and independent pathways produce different antibody isotypes as well as differ in level of somatic mutation of their antibody genes is also suggestive of functionally distinct non-overlapping subpopulations. To date, however, there has been no

direct demonstration of fundamental biochemical differences between B cells activated by anti-Ig verses LPS.

In the experiments previously presented, both of these activation pathways led to B cell support of VSV although anti-Ig and lymphokine stimulated cells consistently synthesize lower levels of PFUs than LPS activated B cells (Table 4). This may argue that characteristics intrinsic to a B cell subpopulation or a consequence of the pathway of activation used to induce the B cells responding to these stimulators accounts for differences in observed PFUs. It is also possible that the diminution in PFUs is due to the counterbalancing action of the different lymphokines contained in the SNs used with anti-Ig stimulated cultures. Lymphokine effects are likely complex, as they could facilitate the full expression of VSV in some cells, have no effect or suppress VSV virus synthesis in others, or modulate VSV expression in the same cell.

Since lymphokines work through receptors, differential expression of receptors on B cells may influence the biochemical capabilities of the cell. The particular array of lymphokine receptors on B cells may in part be constitutively expressed as well as expressed at

particular activation stages, induced by a particular activation pathway, or distributed on distinct subpopulations. Differences in receptor expression have been demonstrated. Activation of murine B cells by co-stimulation with anti-Ig and LPS induces expression of a high affinity receptor for IL-2 that is not induced with LPS activation alone (Zubler et.al., 1987). This expression may reflect either activation pathway or subpopulation differences. Therefore, the potential exists for a variable response by B cells to complex populations of lymphokines, and this ability to respond to particular lymphokines could influence B lymphocytes to support virus infection. The use of VSV expression in B cell populations may be useful as a new model system to study lymphokine effects.

To ascertain how anti-Ig and LPS activated B cells may differ in activity, I determined how the mixture of lymphokines found to enhance VSV replication in anti-Ig stimulated cells would affect virus expression in LPS activated B cells by co-culturing LPS or anti-Ig B cells with various SNs (splenic, Th_1, and Th_2). Table 8, part A clearly demonstrates the suppression of PFU production when lymphokines were added to LPS activated B cells, in marked contrast to the enhancing effects of the same

lymphokines when added to anti-Ig stimulated B cells. Inhibition was seen with all SN but to a variable extent the level of suppression being dependent on the lymphokine source. Splenic and Th_1 derived SNs are completely suppressive, reducing PFU synthesis to levels seen in unstimulated B cells, whereas lymphokines derived from Th_2 clones decrease PFU synthesis 10-fold. These differences are likely due to the presence of a different array of lymphokine receptors on LPS activated cells that now allows signalling leading to virus suppression. It is noteworthy that gamma-interferon, which is known to have anti-viral effects in many systems, is one of a number of lymphokines present in SNs from activated splenic and Th_1 T cells. Assays performed on stocks of splenic SN showed 20-25 units of interferon activity per ml but did not distinguish between alpha, beta or gamma forms. Likewise, interleukin-6, present in Th_2 SN, was initially termed interferon-$beta_2$ as a result of anti-viral activity found in partially purified cytokine preparations, although subsequent studies using recombinant molecules have not demonstrated anti-viral effects (Van Damme et.al., 1987). Lymphokines, in particular interleukin-4 and gamma-interferon, have been shown to have opposing effects on B cell differentiation and antibody production (O'Garra

et.al., 1988), and gamma-interferon has been demonstrated to be a growth factor and not an anti-viral factor for T lymphocytes (Landolfo et.al., 1988). Therefore, while lymphokines with known anti-viral activity on some cell types are present in some of the SN preparations used in these experiments, it is also possible that new unappreciated anti-viral effects of already defined lymphokines or undefined anti-viral lymphokines are present in these SNs, and that the expression of these anti-viral effects in B cells is dependent on the activation pathway inducing specific combinations of receptors.

Is any anti-viral activity associated with constitutively expressed lymphokine receptors or is the suppression seen the result of LPS induced receptors? To answer this question, B cells were exposed to lymphokines for 24 hours, washed and stimulated with LPS for 48 hours to see if there was an effect on their ability to support virus infection. Table 8, part B demonstrates that exposure to various lymphokine mixtures for 24 hours prior to LPS stimulation suppresses PFU synthesis between 4 and 10-fold when compared to B cells activated with LPS alone. Overall, suppression is not as dramatic as that seen upon continuous lymphokine exposure. As the lymphokines were

removed from the B cell cultures prior to the addition of LPS to the cells, suppressive effects may have been minimized by a short action time of suppresive factors. However, I favor the hypothesis that there are differences in the array of receptors for particular lymphokines between resting and activated B cells and that the LPS induced lymphokine receptors are responsible for the anti-viral activity seen. These results demonstrate that the activity of lymphokines on VSV expression is dependent on the B cell population analyzed and is directly affected by the activation pathway through which B cells are induced.

DISCUSSION

These experiments have demonstrated that unstimulated and lymphokine stimulated B cell populations that do not support VSV protein synthesis do support transcription of VSV viral mRNAs (Figure 6). The viral mRNAs synthesized can be translated in a wheat germ translation system indicating that the lack of viral protein synthesis in infected B cell cultures is not due to mRNA defects but rather due to translational control imposed by the B cell populations. Host cell control at the translational level has been observed for viral mRNAs expression in murine mammary tumor virus infected cells

(Vaidya et.al., 1983). Other investigators have shown that expression of two out of five growth-related proteins analyzed in primary lung epithelial cell cultures is controlled at the level of translation but not transcription during cell proliferation and differentiation. Translation of the growth-related genes in these epithelial cell cultures is regulated by growth factors found in serum, and the cellular response to the factors, which influence translation is dependent on the stage of differentiation of the epithelial cell (Clementi et.al., 1990). Likewise, during B cell activation, changes in translational control may occur as the result of different activation signals (lymphokine verses anti-Ig) or at different stages in the activation/ differentiation pathway (G_1 verses S phase of the cell cycle). To further characterize the level of translational control, an analysis of the association of viral mRNA with polysomes is necessary to evaluate whether translation is blocked at elongation or initiation.

Controls at the level of viral transcription are also seen in B cells. The amount of VSV mRNA synthesized in unstimulated B cells is 5 to 10-fold lower than in lymphokine stimulated B cells when equivalent amounts of RNA are analyzed (Figure 6, Table 7). Although

quantitation is not straight forward in this system, a number of possibilities can be envisioned to account for differences in virus transcription levels in non-supportive populations. The general physiological state of unstimulated cultured B lymphocytes may differ from lymphokine activated B cells. These differences may include an overall lower metabolic rate for B cells in the G_o phase of the cell cycle, lower nucleotide pools, inhibitors of transcription, or the generally poor viability of B cells cultured without stimulation. These are all changes that could effect the rate of transcription of both host and viral mRNAs. Perhaps more interestingly, lymphokine exposure may directly enhance transcriptional efficiency in a virus-specific fashion by removing existing blocks to virus transcription or directly enhancing the efficiency of the viral polymerase with specific host factors.

To attempt to address whether the general physiological state of unstimulated B cells after 48 hours in culture is the cause of the lower transcription levels, a comparison of virus transcription levels in freshly isolated and cultured unstimulated B cells can be done. A caveat exists to this experiment; although both of these populations have the same VSV non-supportive

characteristics (Table 4), it is not clear that the population of cells that survive unstimulated culture conditions are representative of the whole starting population. Therefore, possible changes in transcription levels over culture time may represent cell population selection rather than changes in the physiological state. It is also conceivable that only a few B cells in the unstimulated population are carrying out high levels of transcription while other B cells are blocked at other stages of VSV infection, uncoating or transcription. In situ hybridization can be used to address this question by examining both the number of cells and levels of transcription in unstimulated or lymphokine stimulated populations.

The differential activity of lymphokines on B cell populations which have received different activation signals is demonstrated by a number of observations. Lymphokine populations which alone do not activate B cells to support VSV mRNA translation markedly enhance VSV PFU synthesis in B cells co-stimulation with anti-Ig, suggesting that the effect of lymphokines can be dependent on the B cell activation state (Table 4). Second, the lymphokine populations that will enhance VSV PFU synthesis upon co-culture with anti-Ig suppress PFU synthesis when

added to cells activated with LPS suggests that the activator to which the B cells have been exposed markedly influences the B cell response to lymphokines (Table 9 A). Third, pretreatment of B cells with lymphokines prior to LPS activation results in depressed levels of VSV expression when the B cells are subsequently activated by LPS (Table 9). The suppression seen with lymphokine pretreatment is markedly less than that seen when cells are co-cultured with LPS and lymphokines, suggesting that these results are likely a reflection of the unique array of lymphokine receptors displayed by these B cells.

Lymphokines act through receptors that are either constitutively expressed on B cells or induced by activation. If receptors are induced, the pathway of activation could determine the spectrum of receptors expressed as well as differentially regulate the final level of receptor expression. The pathway of B cell activation has been shown to selectively influence the expression of a number of surface glycoproteins which have known or suspected receptor function. Anti-Ig stimulation increases the level of CD5 expression on B cells known to be $CD5^-$ before induction whereas LPS had no effect on CD5 expression (Freedman et.al., 1989; Wortis et.al., 1990 abstract, personal communication) and the expression of

high affinity IL-2 receptors is induced on B cells by co-stimulation with anti-Ig and LPS but not by LPS stimulation alone (Zubler et.al., 1987). These results support the notion that the activation pathway can influence the array of receptors expressed on a single B cell population.

There is another possible level of complication. While both LPS or anti-Ig and lymphokine activation produce antibody secreting cells, it is unknown whether all splenic B cells are capable of responding to either of these stimulators or whether non-overlapping subpopulations of B cells exist which can respond to only one of these activation pathways. If differentially activatable subpopulations of B cells exist, they could express different arrays of receptors constitutively or upon induction. Therefore, not only the process of activation but also the potential activator influences receptor expression. That B cell support for VSV is responsive to these differences presents a working system that allows for a full analysis of lymphokine receptor signal transduction. There are a number of models for B cell activation which can account for the opposing effects of lymphokines on B cell support of VSV that I have noted.

Figure 10 depicts three response schemes that

attempt to incorporate the various action of lymphokines observed in this system. Scenerio A utilizes a single population of B cells which exists as a functionally homogeneous population responsive to either anti-Ig or LPS. Depending on the inducer, a different array of lymphokine receptors, with some potential for overlap (i.e. lymphokine receptor D), is displayed and confers a unique response "phenotype" of the B cells response to a lymphokine mixture. Depending on the biochemical pathway(s) induced in the host following receptor-lymphokine interaction, either enhancement or suppression of virus can occur. Scenario B represents a model where two distinct subpopulations of B cells, each of which is responsive to only one activator. These B cell subpopulations are restricted in the array of induced or constitutive lymphokine receptors they express, thereby facilitating the positive or negative effects on virus replication. Scenario C represents two subpopulations of B cells which can respond to either anti-Ig or LPS, but activation by one pathway enhances the level of expression of certain receptors displayed over the level of expression induced when the other pathway is engaged thus allowing for opposing lymphokine effects.

Models A and C, which assume the ability of the

host B cell to respond to both activators, are consistent with the observation that >90% of B cells undergo blastogenesis upon exposure to either activator while only a fraction of those cells continue to differentiate to antibody secretion. The ability to respond to both anti-Ig and LPS is also consistent with the finding that the addition of anti-Ig to LPS activated B cells inhibits differentiation, but not proliferation of LPS activated B cells (Flahart and Lawton, 1987; Seyschab et.al., 1989). This inhibition of LPS activation by anti-Ig is directed at the transcription of differention-related genes (Flahart and Lawton, 1987).

Further experiments using recombinant lymphokines either alone or in combination with either anti-Ig or LPS activation can be done to define the enhancing or suppressive effects of particular known lymphokines and may also define new functions for these molecules. The possibility also exists that undefined lymphokines present in these SNs may be responsible for some of these activities.

Table and Figure legends

Table 7

Level of viral mRNA synthesis was determined by density scanning techniques on 2 different exposures of film for each probe on B cell populations shown in figure 6. Only those lanes containing 10 ug of total RNA were analyzed and include B cell populations cultured for 48 hours without unstimulation or stimulated with lymphokines (SN), anti-Ig + SN, and LPS. All values determined relative to the unstimulated B cell population signal.

[a] B cell equivalents defined as the number of B cells represented in 10 ug of the total amount of RNA extracted from a known number of B cells. SN, anti-Ig + SN, and LPS values were scored relative to the unstimulated population.

Table 8

[a] In part A, small resting B cells were cultured with the indicated stimulus for 48 hours prior to virus infection. In part B, small resting B cells were cultured for 24 hours with the first stimulus shown, washed and stimulated for an additional 48 hours with LPS.

[b] PFU synthesis per 10^3 cells cultured, mean of

duplicate determinations averaged over two serial dilutions.

Figure 6

Small elutriated B cells were cultured 48 hours with the indicated stimulus prior to infection. Total RNA was extracted 9 hours post-infection for Northern analysis. 10 ug of total RNA was loaded for unstimulated, lymphokine stimulated (SN), anti-Ig + SN, and LPS samples; 2 and 5 ug from 36-65 control cells; and approximately 2 ug from anti-Ig stimulated cells. Probed for VSV mRNAs: panel A - NS mRNA, panel B - G mRNA, panel C - M mRNA, panel D - L mRNA, panel E - N mRNA, and panel F - ethidium stain.

Figure 7

Nothern analysis of total RNA extracted 9 hours post-infection from B cells cultured for 48 hours with the indicated stimulus and probed for actin and GADPH mRNA synthesis. Lane 1 and 2, 2 and 5 ug 36-65; lane 3, unstimulated, cultured B cells; B cells stimulated with lane 4, lymphokines; lane 5, anti-Ig; lane 6, anti-Ig + lymphokines; and lane 6, LPS.

Figure 8

In vitro translation using a wheat germ system of total B cell RNA extracted from: lane 1, unstimulated cultured cells; lanes 2 and 3, lymphokine stimulated, lane 4, anti-Ig + lymphokine; lane 5, LPS stimulated; lane 6, 36-65 control and lane 7, no RNA added. Lanes 8 and 9 are uninfected and VSV infected 36-65 cells, respectively, that were labeled with ^{35}S-methionine *in vivo*.

Figure 9

Immunoprecipitation of translation products with rabbit anti-NDV serum. Protein products from translation of RNA from lane 1, unstimulated cultured B cells; lane 2, lymphokine stimulated; lane 3, anti-Ig + lymphokine stimulated; lane 4, LPS stimulated; and lane 5, hybridoma 36-65. Lanes 6 and 7 are precipitations from uninfected and infected 36-65 cells, respectively, labeled *in vivo*.

Figure 10

Models for B cell activation.

TABLE 7

COMPARISON OF VSV mRNA LEVELS

METHOD OF COMPARISON

A: direct comparison of mRNA

PROBE:	G	M	NS
unstimulated	1.0	1.0	1.0
SN (CDC 35)	5.5	10	5.5

B: mRNA intensity relative to genomic

PROBE:	G	M	NS
unstimulated	1.0	1.0	1.0
SN (CDC 35)	5.0	6.9	5.5

C: mRNA : B cell equivalents[a]

PROBE:	G	M	NS	actin	GADPH
unstimulated	1.0	1.0	1.0	1.0	1.0
SN (CDC 35)	9.5	11.9	9.5	0.7	2.0
anti-Ig + SN	23.9	29.5	32.9	6.5	22.4
LPS	13.6	28.4	17.0	10.8	34.0

TABLE 8

LYMPHOKINES AND VIRUS SUPPRESSION

A

STIMULUS [a]	PFU [b]	STIMULUS	PFU
LPS	2,160	ANTI-Ig	36
LPS + Sp SN	3	ANTI-Ig + Sp SN	450
LPS + Th2 SN	230	ANTI-Ig + Th2 SN	908
LPS + Th1 SN	1	ANTI-Ig + Th1 SN	264

B

LPS -> LPS	2,280
Sp SN -> LPS	668
Th2 SN -> LPS	160
Th1 SN -> LPS	626

FIGURE 6

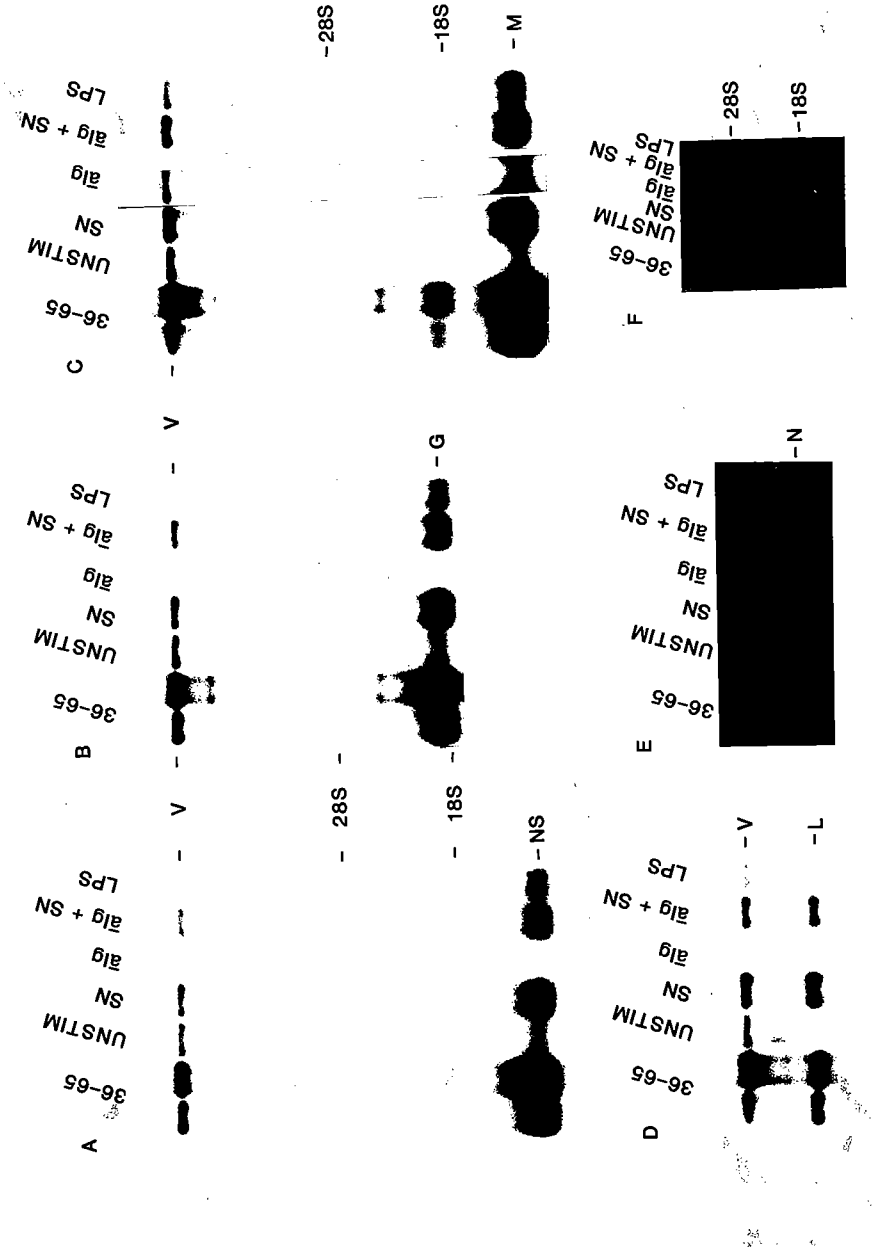

A
36–65
UNSTIM
SN
alg
alg + SN
LPS
– V
B
36–65
UNSTIM
SN
alg
alg + SN
LPS
– V
C
36–65
UNSTIM
SN
alg
alg + SN
LPS
– V
D
36–65
UNSTIM
SN
alg
alg + SN
LPS
– V
– L
– NS
– 28S
– 18S
– G
– 28S
– 18S
– M
E
36–65
UNSTIM
SN
alg
alg + SN
LPS
– N
F
36–65
UNSTIM
SN
alg
alg + SN
LPS
– 28S
– 18S

FIGURE 7

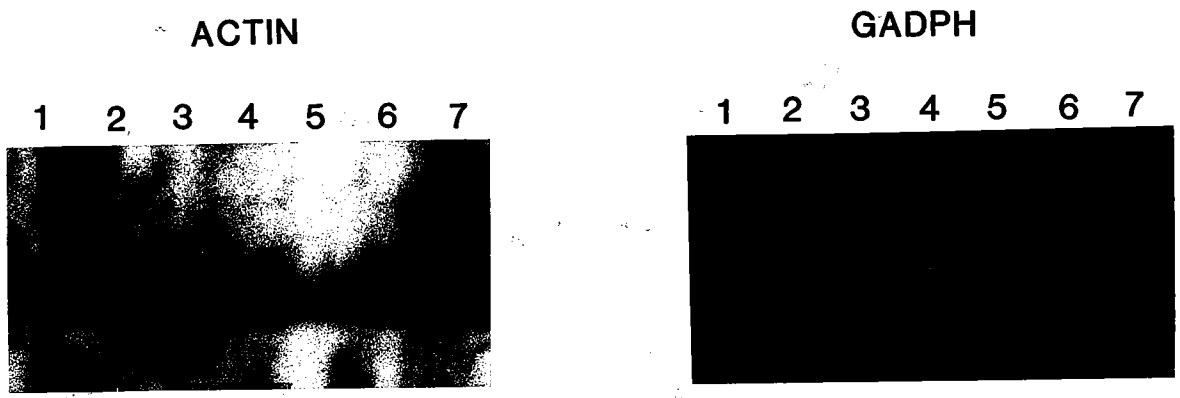


FIGURE 8

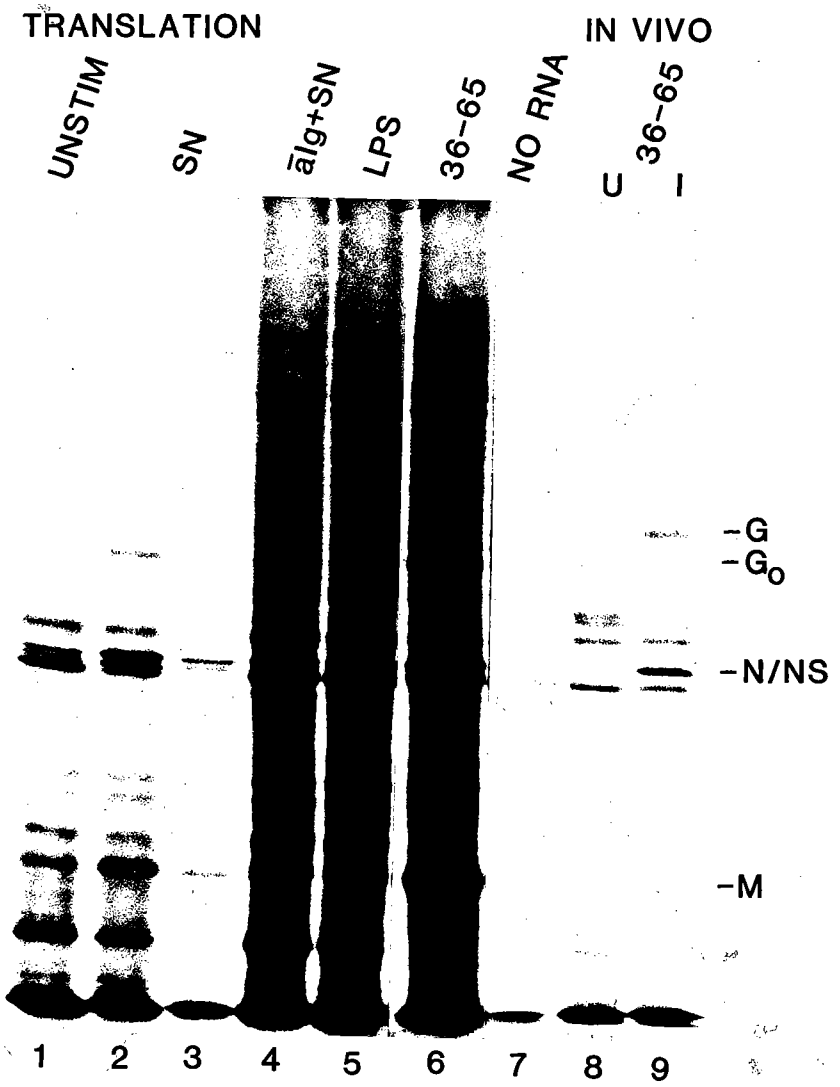

TRANSLATION
IN VIVO
UNSTIM
SN
ālg+SN
LPS
36-65
NO RNA
U
36-65
I
-G
-G$_O$
-N/NS
-M
1
2
3
4
5
6
7
8
9

FIGURE 9

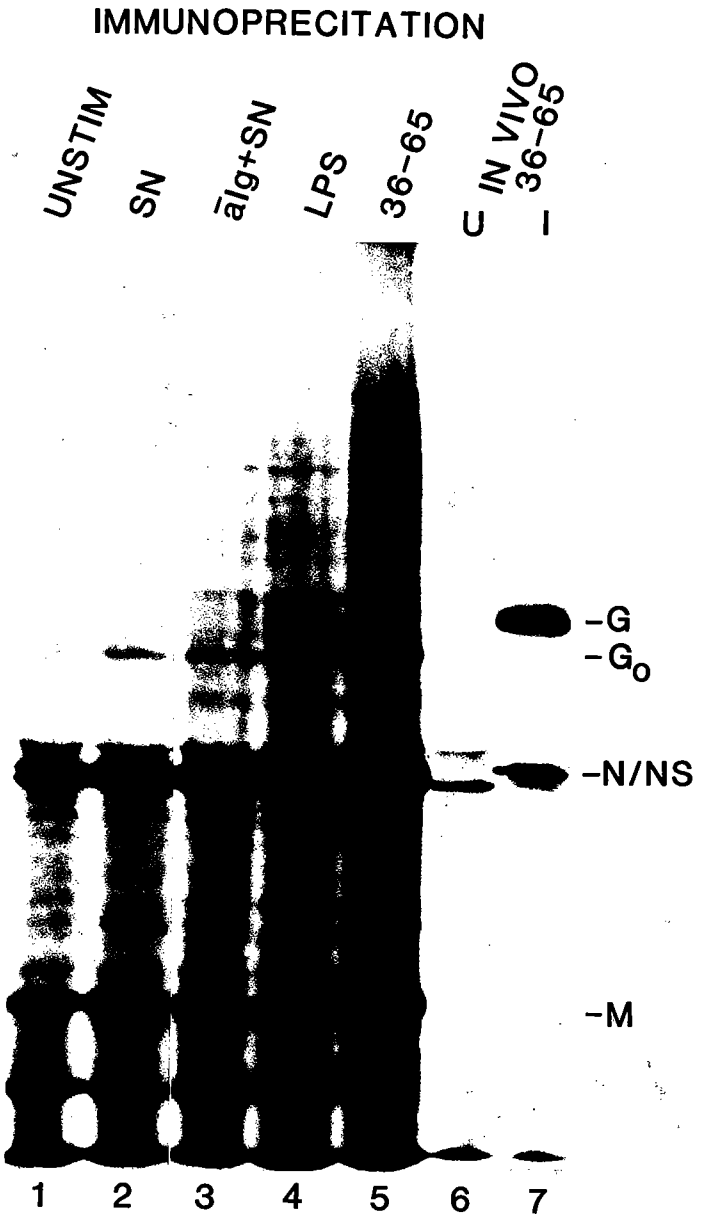
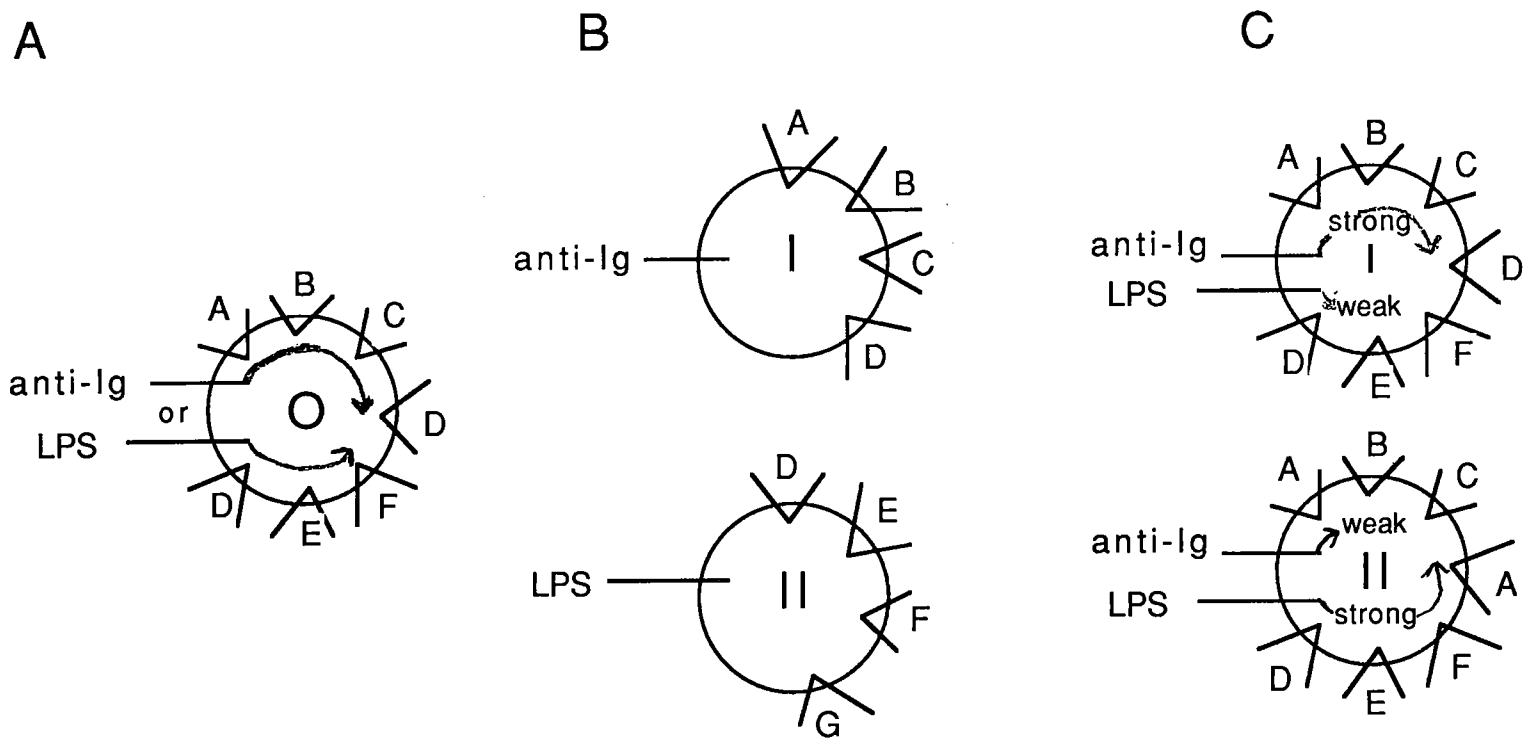

IMMUNOPRECITATION
UNSTIM
SN
āIg+SN
LPS
36-65
U IN VIVO 36-65
I
-G
-Go
-N/NS
-M
1
2
3
4
5
6
7

FIGURE 10



CHAPTER 3

VSV expression in partially supportive B cells

INTRODUCTION

B cells activated with anti-Ig, at concentrations which allow entry into the S phase of the cell cycle, support the synthesis of VSV proteins (Figure 3) and an increased number of infectious centers but produce few PFUs (Tables 4 and 5). These characteristics of VSV infected anti-Ig stimulated B cells are phenotypically similar to those found when other cell types undergo persistent infection with viruses (Younger and Preble, 1980). Persistently infected cells may serve as reservoirs for virus, constantly shedding low levels of infectious virus particles or possibly converting to an acute lytic state upon activation under the appropriate conditions. For example, human B lymphoctes persistently infected with EBV can be induced into a lytic infection by stimulating the B cells with anti-Ig (Crawford and Ando, 1986).

Mechanistically, persistent viral infections may be due to host imposed restrictions on virus replication and/or the selection of virus variants. Virus variants may bear mutations in one or a number of viral genes which

can result in loss of expression of a viral protein or the alteration of protein function, producing such changes as the predominant synthesis of defective interfering virus particles or the inappropriate localization of viral proteins (Youngner et.al., 1978; Youngner and Preble, 1980). In vivo, virus variants causing persistent infections must escape immune system detection, without being cytopathic for the host cell. Alteration of protein localization during persistent infection has been demonstrated in newborn mice infected with lymphocytic choriomeningitis virus. During this persistent infection viral glycoproteins are not found on the surface of infected cells 30 days after infection, thus aiding in the escape from immune system surveillance. During the first two weeks of LCMV infection the expression of viral glycoproteins on the host cell surface was similar to that of acutely infected cells, suggesting that either the immature immune system of newborn mice or some other mechanism for eluding immune surveillance, such as short lived immunological tolerance, permitted establishment of the persistent infection (Oldstone and Buchmeirer, 1982). In vitro, the expression of the persistently infected phenotype usually requires the outgrowth of a clone or clones of cells originally infected with a viral variant

produced during an acute infection (Youngner et.al., 1978; Welsh and Oldstone, 1977; Oldstone and Buchmeier, 1982).

Host imposed restriction on virus infection is seen in the failure of host cells to support a number of primary viral infections. Inhibition of replication of viral genomic RNA has been observed during VSV infection of non-permissive rabbit cornea cells (Hamilton et.al., 1980) and the human B cell line, Raji (Johnson and Herman, 1984). Persistent VSV infections are also readily established in EBV-positive but not EBV-negative human B cell lines by an as yet unknown mechanism that does not apparently involve viral genomic replication (Creager et.al., 1982). Kinetics suggest that the phenotype of persistently infected cells observed with VSV infection during primary infection (24 hours post-infection) of anti-Ig stimulated B cells is likely the result of host cell imposed restrictions on VSV infection rather than selection of virus variants. Host B cell control of the outcome of VSV infection is further supported by preliminary evidence showing that the addition of lymphokines (Th_2 SN) to VSV infected B cells after their stimulation for 48 hours with anti-Ig alone results in high level PFU synthesis. VSV infected anti-Ig stimulated B cells gave 38 PFU per 10^3 cells when cultured without

additional stimulus and produced 1300 PFU per 10^3 cells when Th_2 SN was added to the infected cell cultures (B cells co-cultured with anti-Ig and lymphokines produced 780 and 1130 PFU per 10^3 cells with and without SN, respectively). These results demonstrate that further activation of the infected anti-Ig stimulated B cells supported by lymphokines induces cellular changes which can then support VSV PFU synthesis at a level equivalent to that observed when B cells are co-stimulated with anti-Ig and lymphokines prior to infection.

Effects on virus infection or replication that are the consequence of either host cell restrictions or mutational events can result in 1) a lower level of viral transcription in persistently infected cell lines when compared to lytically infected cell lines (Creager et.al., 1981) or selective impairment of a particular mRNA transcript due to lower transcription levels or increased degradation (Vaidya et.al., 1983), 2) deficiencies in viral protein synthesis or inappropriate cellular localization of viral proteins (Vaidya et.al., 1983; Welsh and Buchmeier, 1979; Oldstone and Buchmeier, 1982; van der Zeijst et.al., 1983), 3) inhibition of replication of the viral genome (Nowakowski et.al., 1973; Johnson and Herman, 1984; Hamilton et.al., 1980), and 4) defective maturation

of virus particles (Youngner and Preble, 1980; Meltzer et.al., 1990). Anti-Ig stimulated B cells support the synthesis of the complete array of VSV proteins and low levels of PFU synthesis suggesting that the block to high level PFU synthesis is at a point beyond viral protein translation, such as inappropriate cellular localization of viral proteins, genomic replication, virion assembly or maturation. Although previous experiments demonstrated no apparent deficit in a particular viral protein when infected cell lysates were immunoprecipitated with rabbit anti-VSV serum (Figure 3), these precipitations were not truly quantitative, making it possibile that selective defects in protein synthesis could produce the phenotype of low PFU synthesis. Limited protein synthesis could result from a generalized or specific decrease in VSV mRNA transcripts or by host cell translational control. The following experiments address the first mechanism of persistence previously discussed by determining the level of viral mRNA synthesized in anti-Ig stimulated B cells.

RESULTS

VSV mRNA transcription in anti-Ig activated B cells

A possible restriction on PFU synthesis in VSV infected anti-Ig stimulated B cells may be a limitation on

the transcription of either some or all VSV genes. This would limit the amount of required viral protein(s) leading to low levels of VSV replication. To address this question the synthesis of VSV mRNA's was analyzed by Northern blotting of total RNA extracted from lymphokine, anti-Ig, anti-Ig plus lymphokine, and LPS stimulated B cell populations. The results of this analysis, shown in figure 11 (A - ethidium stain, B - L mRNA, C - G mRNA, D - M mRNA, E - NS mRNA, and F - N mRNA), shows that probes for all viral mRNAs readily hybridize with mRNA extracted from anti-Ig stimulated B cells. No apparent deficit in any one viral mRNA or in the overall amount of viral mRNAs, assessed by density scanning, is observed when the viral mRNAs from anti-Ig stimulated B cells are compared to mRNAs from permissive B cell populations (anti-Ig and lymphokines or LPS). Moreover, no differences were noted when hybridization was compared with non-permissive lymphokine stimulated B cells.

The pattern of viral protein synthesis observed with ^{35}S-methionine labeled extracts of VSV infected anti-Ig stimulated cell cultures (figure 3, lane 2) is consistent with the production of VSV mRNAs. Moreover, no obvious deficit in the synthesis of any viral protein is seen when limiting amounts of infected cell lysates are

immunoprecipitated with excess antibody to VSV proteins. Although not precisely quantitative, this technique provides an overall assessment of viral protein synthesis, and a significant deficit in synthesis of any viral protein would have been detected. Therefore, it is unlikely that anti-Ig stimulated B cells limit VSV replication by translational control as was seen for unstimulated and lymphokine stimulated B cell populations.

These findings suggest that the low level of PFU production by B cells activated with anti-Ig is most likely due to a restriction on VSV infection at a point beyond primary transcription and translation, and suggests the need for other analytical approaches to determine the site of restriction in PFU synthesis in anti-Ig stimulated B cells. These approaches are presented as the discussion.

DISCUSSION

VSV genomic replication in anti-Ig activated B cells

Replication of VSV genomic RNA is required for both the amplification of infection within a cell as well as the synthesis of virions. Replication involves the synthesis of positive-sense copies of the viral genome, which are then used as templates for the synthesis of negative-strand viral RNA, which can be packaged into

progeny virions. The process of replication is dependent on ongoing viral protein synthesis, in particular the VSV N and NS proteins (review: Wagner, 1990). In vitro replication studies also suggest the involvement of host-cell factor(s) in replication as nucleocapsids and cytoplasmic protein fractions from VSV infected cells were most efficient for in vitro replication (Peluso and Moyer, 1988). Restriction on VSV RNA replication has been observed in the non-permissive infection of rabbit cornea cells (Hamilton et.al., 1980) and in some B lymphoblastoid cell lines (Nowakowski et.al., 1973; Johnson and Herman, 1984). Therefore, it is possible that the synthesis of either positive or negative sense replicative forms of VSV genomic RNA is deficient in anti-Ig activated B cells resulting in the low level PFU synthesis.

In primary B cells, viral RNA replication can be directly assessed with Northern analysis and the use of probes specific for either positive or negative sense VSV genomic RNA species. I have taken the initial steps to approach this question by cloning a portion of the VSV G cDNA clone into a Bluescript vector so that RNA probes for either positive or negative sense strands of VSV can be prepared by in vitro transcription using T3 or T7 RNA polymerases. Synthesis of positive-strand virion forms in

anti-Ig stimulated B cells can be directly examined and compared with synthesis levels of positive sense RNA in the fully supportive B cell populations. The analysis of negative-strand genomic RNA synthesis requires that the level of input (infecting) genomic RNA, which is of negative sense, be used as a measure of background in order to assess increases in this RNA species. Input VSV genomic RNA will be quantitated by hybridization with the negative-strand specific probe to RNA samples from B cells harvested one hour post-infection. As the level of negative-strand synthesis increases over the time of infection, a comparison of the input genomic RNA with the amount of negative-strand RNA found at later times in infection will be used for this assessment. Because the B cell populations used for these analyses vary in the number of cells producing virus, assessed by infectious center assay (Tables 4, 5), as well as in the amount of RNA per B cell, cellular RNA content increases during activation, a correction must be included in the analysis. A comparison of the level of viral genomic RNA synthesis relative to the number of B cells producing infectious centers in the same B cell population or a comparison based on the number of B cells represented by the amount of RNA analysis by hybridization (calculated according to

the total amount of RNA extracted for the given number of B cells in the population extracted) must be done to analyze population differences. This analysis should indicate if the low level of PFU production in anti-Ig stimulated B cells can be attributed to restrictions on the synthesis of positive or negative genomic forms.

The possibility also exists that anti-Ig stimulated B cells preferentially synthesize and package defective rather that full length genomes. Because defective interfering particles (D.I) are not infectious, the production of defective interfering (D.I.) particles could explain the low level of PFUs detected from anti-Ig infected B cells. While D.I. particle synthesis usually increases over time in a persistent infection or is observed early in infection when infections are done at high multiplicities of infection or with virus populations containing high levels of D.I. particles (Youngner and Preble, 1980; Youngner et.al., 1978), anti-Ig stimulated B cells may promote D.I. synthesis either by failing to provide a required host cell factor for stabilization of the replication complex or by directly inhibiting synthesis of full length genomic RNAs by destabilizing the viral polymerase during replication. Since D.I. genomes are packaged into virus particles, an analysis of the RNA

species in particles released into the culture SN from infected anti-Ig stimulated B cells can be done to examine this question. D.I. genomes contain incomplete viral RNA sequences, 50% or less of the standard genome that include simple deletions or snap-back forms which contain sequences complementary to both positive and negative genomic sense RNA. Because virion RNA from infectious virus particles should be of uniform size and polarity, the finding of packaged, smaller size RNAs or RNA species containing sequences complementary to positive- and negative-sense probes would suggest synthesis of defective forms. To examine this possibility, viral RNAs produced in anti-Ig stimulated B cells can be directly labeled with ^{3}H-uridine, released particles pelleted from the culture SN and examined for size by electrophoresis on denaturing urea-acrylamide gels. Alternatively, unlabeled RNA can be extracted from SN pellets and subjected to Northern analysis. The use of probes specific for either positive or negative sense genomic RNA would allow the detection of packaged RNA species of different sizes as well those which contain sequences complementary to both senses of RNA demonstrating synthesis of the snap-back form of D. I. particles.

<u>VSV protein localization in anti-Ig activated B cells</u>

Viral protein maturation or localization in anti-Ig stimulated B cells may not be appropriate for formation of VSV particles, serving as an alternative or additional point of restriction on VSV infection. The VSV-G glycoprotein requires oligomerization in the endoplasmic reticulum for efficient transport to the plasma membrane. This requirement has been established by demonstrating that G protein mutants, incapable of forming oligomers, are either transported inefficiently or not at all (Kreis and Lodish, 1986; Doms et.al., 1987; Doms et.al., 1988). In addition, other VSV proteins are required at sufficient levels for virus replication, e.g. enough VSV N and NS proteins must be available to associate with and cover the viral RNA during genomic replication. The VSV-M protein is involved in virion maturation by binding to replicated nucleocapsid structures in the cell cytoplasm. This complex then migrates to the cell membrane, and the M protein mediates association with membrane areas containing the G protein (review: Wagner, 1990). Therefore, the low PFU production seen in anti-Ig stimulated B cells may reflect a host-imposed inability to support or a direct inhibition of viral protein maturation or complex formation.

Oligomerization of the G glycoprotein requires the

participation of a host cell derived component. The host heavy chain binding protein (BiP, also called glucose regulated protein 78, GRP78, Munro and Pelham, 1986) is involved in the process of protein oligomerization of the native VSV G-glycoprotein (de Silva et.al., 1990). BiP/GRP78 is a member of a family of stress proteins and its expression is induced during B cell activation (Wiest et.al., 1990), glucose starvation (Shiu et.al., 1977), virus infection (Collins and Hightower, 1982; Garry et.al., 1983; Sarnow, 1989), and with calcium ionophores (Watowich and Morimoto, 1985). This cellular protein is required in B cells for the oligomerization of heavy and light chains during antibody formation (Hendershot et.al., 1987). It is noteworthy that the differentiation of B cells to high level synthesis and secretion of immunoglobulin, supported by the addition of lymphokines to anti-Ig stimulated B cells, are also the additions necessary for increased VSV PFU synthesis when B cells are infected. Therefore, it is possible that the level of BiP in anti-Ig stimulated B cells may be insufficient for efficient VSV-G glycoprotein assembly.

Cellular proteins besides BiP are also induced during B cell activation (Wiest et.al., 1990) and may be involved in enhancing or inhibiting viral protein

localization or assembly of virions. Host cell proteins, also members of stress protein families (hsp and grp proteins), have been identified that are involved in assembly and transport of protein complexes or associated with cell receptors (Young, 1990). Enhanced expression of some of these stress proteins, even during virus mediated shut-off of other host cell protein synthesis, as well as the co-precipitation of viral proteins with stress proteins have been detected in virus infected cells, suggesting that these cellular proteins can play an important role during virus infection (Collins and Hightower, 1982; Garry et.al., 1983; Sarnow, 1989). For example, the VSV N protein has been coprecipitated with anti-hsp90 antibodies from infected chick embryo cells (Garry et.al., 1983). Therefore, viral protein maturation or localization can be affected at a number of levels by changes in protein expression during the B cell activation process.

To begin to address the question of protein localization in B cell populations, the expression of the VSV-G protein on the cell surface will be examined. Surface staining, using a monoclonal antibody specific for the VSV-G glycoprotein (kindly provided by Dr. C. Reiss) will be used to assess whether anti-Ig stimulated B cells

are deficient in surface expression of the G protein. A failure in surface staining or low level of staining compared to permissive B cell populations would suggest that there may be a problem in protein transport or oligomerization, possibly involving expression of BiP in anti-Ig stimulated B cells. Immunoprecipitation of the G protein, using the monoclonal antibody, from infected cell lysates followed by SDS-gel electrophoresis under non-reducing conditions can be done to determine whether protein oligomerize of G is occuring. Since BiP is required for VSV-G protein association, a failure to oligomerization would suggest that anti-Ig stimulated B cells are deficient in this cellular protein. The synthesis of BiP in anti-Ig stimulated B cells and its association with VSV-G protein during infection can be examined by immunoprecipitation with anti-Ig antibody or anti-BiP antibody (kindly provided by L. Hendershot). If either the rate or overall synthesis of BiP is low in anti-Ig stimulated B cells, longer ^{35}S-methionine labeling times or even labeling of cellular proteins prior to infection may be required in order to label sufficient BiP for this analysis.

A general analysis of other cellular stress proteins expressed during different B cell activation

states as well as those cellular proteins found associated with viral proteins, such as VSV N with hsp90, may provide insights into restrictions on support of VSV infection by B cells. Previously, infected cell lysates were prepared following alkylation with iodoacetamide, which may have disrupted protein-protein interactions. Therefore, immunoprecipitation with anti-stress protein antibodies and/or anti-VSV antibodies from cell lysates prepared without alkylation may be useful for the detection of interactions between B cell proteins and VSV proteins. Stress proteins bind ATP so that a method for examining their general expression in B cells exposed to different activators can be carried out by using affinity chromatography followed by gel electrophoresis of labeled cell lystaes.

VSV particle maturation in anti-Ig activated B cells

The proper maturation of virus particles may be the limiting step in VSV infection of anti-Ig stimulated B cells. Virus particles may not be formed, may be formed properly but not released, or formed improperly. Each of these possibilities can be directly addressed experimentally.

Previous assays for PFU synthesis have been done by simply collecting SNs from cultured cells. An initial

examination of PFU synthesis from cell populations where viral SNs are collected with and without freeze thawing of the B cells may indicate if the problem is associated with release of virions from the cell surface. Assembly of viral components, such as an inability of the nucleocapsid to associate with the plasma membrane, could result in a lack of particle formation. This would most likely result from interference by components of the host cell membrane causing inhibition of maturation or release of mature virions. An electron microscopic examination of anti-Ig activated B cells can be done to see if assembly of virion nucleocapsids is occuring at the cell membrane.

If virus particles are produced by infected anti-Ig stimulated cells, the amount of glycoprotein incorporated into the virion, protein composition of the nucleocapsid, or some affect of the host cell plasma membrane components (lipid or protein) may affect subsequent PFU detection. Changes in virion composition, such as limited numbers of viral glycoproteins, have been shown to alter virulence, affect viral tropism and target cell susceptibility for a number of viruses, including HIV (Meltzer et.al., 1990) and NDV (Sheehan et.al., 1987). To look at this possibility, an analysis of the protein composition of virions can be done by continuous labeling

of proteins during B cell infection with subsequent pelleting of released virus particles and analysis by SDS-gel electrophoresis. A comparison of protein composition of virions from anti-Ig stimulated B cells and permissive B cell populations may indicate if this is a point of restriction.

It is also possible that the rate of virion assembly in anti-Ig stimulated B cells is slower than in anti-Ig plus lymphokine stimulated cells. The addition of lymphokines to anti-Ig activated B cells does induce higher rates of antibody synthesis and may induce critical host cell proteins required for efficient transport, assembly of virion components, or alterations in membrane fluidity. As the assessment of PFU synthesis has been done at 24 hours post-infection, any rate differences might give the impression of lower synthesis. Examination of PFU production up to 72 hours post-infection or even an analysis of the replication of genomic RNA over a prolonged period of time may indicate if this is cause of low PFU synthesis.

In summary, VSV infection of anti-Ig stimulated B cells presents a pattern of virus expression similar to persistently infected cells, i.e. viral protein synthesis without PFU production. Analysis of VSV infection in B

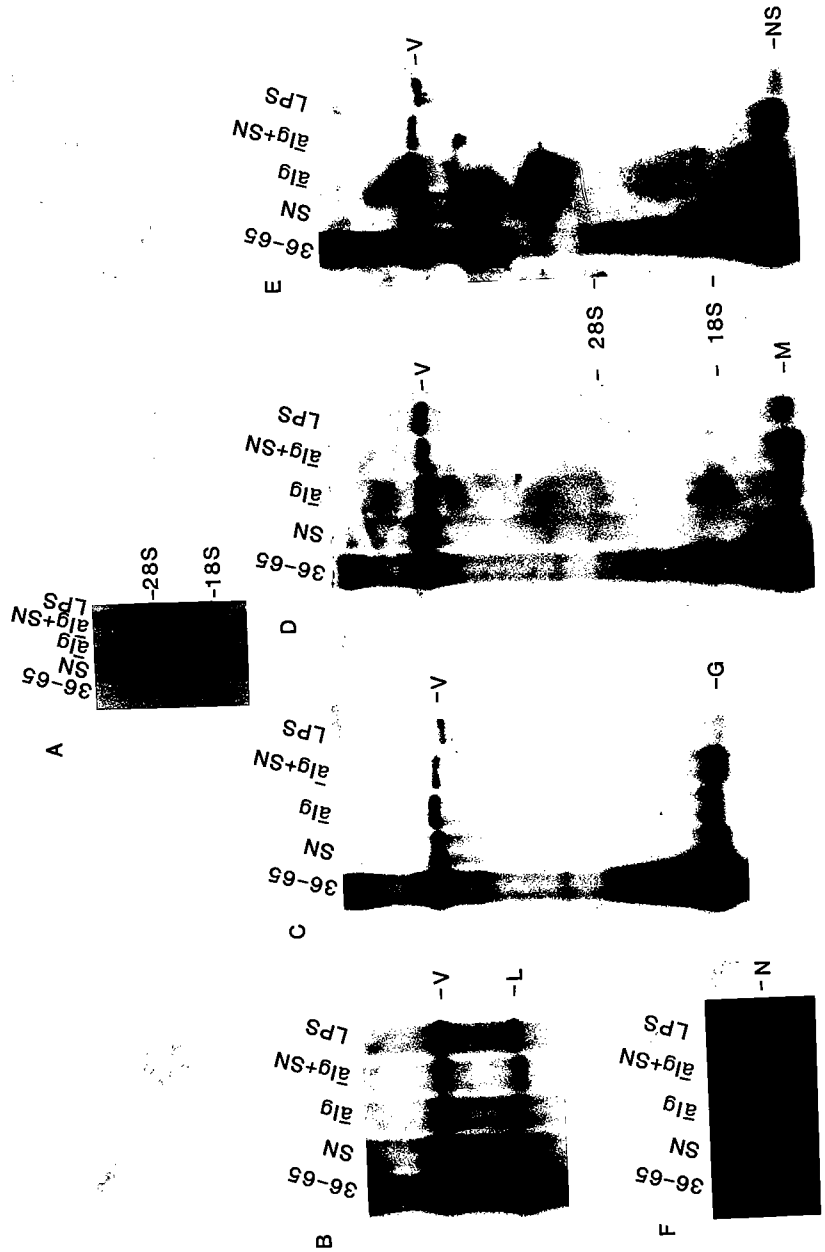
cells in this activation state may present a new model for systematically analyzing the influence of possible host cell-derived factors on virus infection in primary cells where the outcome of infection is host controlled and reliably obtained.

Figure legend

Figure 11

Total RNA was extracted 8 hours post-infection from the control B cell hybridoma 36-65 and from B cell populations stimulated for 48 hours prior to VSV infection with the indicated stimulus (lymphokines (SN), anti-Ig, anti-Ig + lymphokine, and LPS). Samples of RNA, 2ug from 36-65 and 10ug from the B cell populations, were separated by agarose-formaldehyde gel electrophoresis, processed (see methods) and probed for VSV mRNAs: Panel B - L mRNA, panel C - G mRNA, panel D - M mRNA, panel E - NS mRNA, and panel F - N mRNA. Panel A is the ethidium stain.

FIGURE 11



CHAPTER 4

NDV INFECTION OF B LYMPHOCYTES

INTRODUCTION

Experiments previously described have defined a system suitable for the analysis of the activation requirements necessary to render B lymphocytes capable of supporting a virus infection. These data showed multiple restrictions on a productive VSV infection that were dependent on the activation signal(s) a B cell population had received. It was of interest to determine if the conditions established for a productive VSV infection were equivalent to those necessary to support other viruses, as it was felt that such a comparison could provide new insights into the process(es) by which lymphocytes are rendered into permissive hosts for virus infection. NDV, a member of the paramyxovirus family of negative-stranded RNA viruses which includes parainfluenza, mumps, measles, and respiratory syncitial virus, was chosen for the first comparative analysis.

The NDV virion, like VSV, is composed of a lipoprotein envelope surrounding the nucleocapsid core. The single stranded RNA genome encodes six viral proteins, undergoes cytoplasmic replication with virion assembly at

the host cell plasma membrane, and is dependent on host cell factors for these processes (general review: Kingsbury, 1990). Both NDV and VSV undergo permissive, lytic infections in a number of the same cell types, such as primary chick embryo fibroblasts, CHO cells, the myeloma SP2/0, and the hybridoma 36-65; all have been used as control target cells in our experiments. Since NDV lytically infects chick embryo fibroblasts, the same infectious center and PFU assay systems set up for VSV can be used to assess B cell support for NDV infection.

Although both paramyxoviruses and rhabdoviruses have similar replication patterns, some important differences have been observed. One distinction between NDV and VSV infection is the route of entry of the virus. The NDV F surface glycoprotein mediates fusion between the virus and cell membranes. This releases the nucleocapsid directly into the cell cytoplasm, as opposed to the requirement for endocytosis of the VSV virion. Spread of infection by the paramyxovirus family can occur not only through the dissemination of infectious particles, but also by fusion between infected and uninfected cells mediated by the viral F glycoprotein (Gallaher and Bratt, 1974). Viral protein mediated fusion results in syncitia formation within the host and is in part responsible for

the viral cytopathology associated with a number of viral diseases induced by paramyxoviruses (NDV, respiratory syncitial virus, measles), as well as other viruses, including coronaviruses (mouse hepatitis virus), and retroviruses (HIV).

A second difference found between NDV and VSV involves the surface expression of viral glycoproteins. Experiments using polarized cells demonstrated that the rhabdovirus glycoproteins were expressed on the basolateral cell surface, whereas paramyxovirus glycoproteins were expressed on the apical surface (Rodriguez-Boulan and Sabatini, 1978). Other studies have shown that, in the same cell type, the rate of surface expression of the NDV and VSV glycoproteins differs because of differences in transit times between the RER and the trans golgi (Morrison and Ward, 1984). These findings suggest that different cellular pathways and/or host cell factors may be involved in the maturation of these proteins as well as the virus particles. Hypothetically, in B cells, these cellular pathways may be affected by the spectrum of inductive signals a B cell has received and result in differences in the ability of B cells to support these different viruses.

Using the previously defined system for activating

B cells to different points in their activation pathway, I have assessed their ability to support NDV infection, applying the same criteria of infection previously employed for VSV. I find that the B cell's ability to support a productive NDV infection differs markedly from the requirements necessary to support VSV.

RESULTS

NDV infection of B cell enriched populations

In initial experiments freshly isolated murine splenic B cell enriched populations were activated with various stimuli and were then tested for their ability to support NDV infection and replication. These B cells, >95% surface Ig^+ by fluorescent staining, were unelutriated and represented a continuum of activation states due to *in vivo* stimulation. Table 9 shows the number of infectious centers per 10^3 cells in B cell populations infected after exposure to different activators in culture for 24, 48, and 72 hours. B cells cultured with no additional stimulus for 24 and 48 hours produced 31 and 59 infectious centers, respectively. These values are used as background levels in this experiment. This background level of infectious center

formation in B cell enriched populations, also observed for VSV infection (Table 3), is likely due to infection of B cells previously activated in vivo. Cells cultured in the presence of splenic SN derived lymphokines show a 2-fold increase over background levels at these time points. This increase in NDV infectious centers may result from lymphokine induced B cell progression into the G_1 phase of the cell cycle and/or result from the ability of lymphokines to support further differentiation of B cells which have been previously activated in vivo. It is also possible that other contaminating cell types in the cell mixture, such as macrophages, polymorphonuclear neutrophils, etc., are induced to respond more effectively to NDV infection after lymphokine exposure or themselves provided lymphokines that could alter the B cell response. B cells stimulated with anti-Ig or anti-Ig and lymphokines (splenic SN) demonstrate the greatest increase. At 48 hours, anti-Ig stimulated cultures are increased 4-fold (236 infectious centers) over background levels while anti-Ig and lymphokine stimulated B cells show a 6-fold increase (347 infectious centers per 10^3 cells plated) over background. In contrast, the number of infectious centers observed in LPS activated cultures was only 2-fold over background, i.e. 125 per 10^3 cells plated. The

number of cells producing infectious centers remained relatively constant between 48 and 72 hours for anti-Ig and LPS stimulated populations. The NDV permissive murine B cell line, SP2/0, produced 420 to 452 infectious centers per 10^3 cells plated when used as a control in these experiments (Table 9 legend).

These data are consistent with an increased ability to support a productive NDV infection, at least as determined by infectious center formation, as B cells are stimulated further along their activation/differentiation pathway. While these experiments demonstrate that B cells can be activated to support increased numbers of NDV infectious centers, they do not define specific activation requirements due to the presence of endogenously activated B cells and other cell types in the partially purified populations. To better determine activation requirements for splenic B cell support of NDV infection, B cells, size fractionated by elutriation, were used for all subsequent experiments.

NDV infection of purified small B cell populations

Freshly isolated small resting B cells were exposed to NDV on the day of isolation (Table 10 A) in order to fully assess susceptibility to NDV. This B cell population produced 27 NDV infectious centers per 10^3

cells plated. Maintenance of these cells in culture for 24 and 48 hours without further stimulation demonstrated a slight increase in number of infectious centers, 52 and 38 per 10^3 cells plated, respectively. In these unstimulated B cells the "background levels" for NDV contrast significantly with the background levels observed for VSV (between 1 to 4 I.C./10^3 cells plated, Tables 4 and 5) when susceptibility to both viruses was assessed in the same B cell population, clearly suggesting an intrinsic difference in small B cell support for these two viruses. Stimulation with lymphokines contained in splenic SN demonstrated no increase over background at 24 hours and a 2-fold increase over background at 48 hours, up to 86 infectious centers. B cells stimulated for 48 hours with anti-Ig or LPS prior to infection with NDV demonstrate 5 and 3.5 fold increases in infectious centers over background to 210 and 140 infectious centers, respectively.

To determine the full spectrum of responses to B cell activators, small B cells were cultured prior to NDV infection with lymphokines from T helper cell lines, which produce both different and more defined collections of lymphokines upon stimulation with Con A (Mossman et.al., 1986) from those found in splenic Con A SN (Table 10 B).

B cells stimulated with these populations of lymphokines produced a 2-fold increase in infectious centers over background, the same as that observed for B cells stimulated with splenic SN derived lymphokines. Anti-Ig and LPS demonstrate 3 and 4-fold increases in infectious centers, respectively.

Data from these experiments with purified B cells indicate that after 24 hours of stimulation there is no consistent increase in NDV infectious centers over the background level for any stimulus tested. After 48 hours of stimulation the number of NDV infectious centers increases 2-fold upon stimulation with lymphokines from a variety of sources. This lymphokine effect, in general, may be more apparent than real, as there is little change in number of infectious centers between 24 and 48 hours for the lymphokine stimulated B cells, while most of the change is seen relative to the background level from unstimulated B cells which drops 2-fold (Table 10). These results with NDV infection of lymphokine stimulated B cells are comparable to those observed for VSV infection, as lymphokine stimulation did not increase the number of cells producing VSV infectious centers (Tables 4 and 5). Activation into S phase of the cell cycle with anti-Ig further enhances plaque formation up to 5-fold over

background and is as effective as LPS stimulation. The results of these experiments on B cell support of NDV infection contrast at a couple of levels with B cell support of VSV where the background level of infectious center formation was only 1 to 5 per 10^3 cells plated and LPS was more effective than anti-Ig stimulation.

In addition to assaying infectious center formation, the production of infectious NDV particles was measured from culture SNs obtained 24 hours after initiation of infection in the same populations of B cells. Low levels of PFUs were produced under all stimulating conditions; less than one PFU per infectious center was observed when culture SNs from infected unstimulated, lymphokine stimulated, or LPS activated B cells and up to 1-2 PFUs per infectious center was seen with anti-Ig stimulated B cells (Table 10 B). Infection of murine B cells in general is not apparently the problem for support of NDV, because infection was found to proceed normally in the B cell hybridoma, 36-65, and myeloma, Sp/2, which produce 15-25 PFU/I.C.

By the characteristic of PFU production, NDV infection of B cells differs once again from VSV infection in that the level of VSV PFU synthesis increases proportionally to the "inductive stimulus", as PFU

production in LPS activated B cellls is equivalent to that found in permissive cells. Whereas activated B cells support an increase in numbers of NDV infectious centers, few PFUs are produced or detected from any culture SNs. The course of NDV infection is locked into the same mode found in anti-Ig stimulated VSV infected B lymphocytes and does not transit beyond this step.

A number of possibilities may account for the lack of PFU detection under conditions of infectious center formation. Infectious centers may result from NDV F protein mediated fusion between the B cell and the permissive indicator monolayer. This fusion event, termed fusion from within, does not require the synthesis or release of virions (Gallagher and Bratt, 1976). Fusion of B cells with the permissive indicator cell could be mediated by newly synthesized F protein and would allow infectious plaque formation to be observed even if B cells are defective at some stage in NDV replication or maturation of virus particles (stability or release) because the infection would be fully supported by the permissive cell monolayer. The possibly that fusion is mediated by residual F protein from the infecting virus located on the B cell surface is unlikely due to post-infection treatment of B cell cultures with anti-NDV serum

as well as by the fact that increases in infectious center formation are observed with B cell activation, a finding inconsistent with fusion mediated by input virions. Another possibility which could account for the lack of PFU detection is that the proximity of a supportive B cell and the indicator cell may facilitate detection of virus particles by infectious center formation if rebinding of released virions is occuring in the infected B cell cultures, thus artifically lowering PFU numbers found free in culture SNs.

To begin to examine the question of NDV PFU synthesis, a further examination of NDV particles released into culture SN was performed. For NDV particles to be infectious, the newly synthesized viral F glycoprotein in the F_o (inactive) form must be cleaved to the F_1 (active) form by a cellular enzyme. If this enzymatic activity is inefficient or absent in a host cell, non-infectious virus particles may be produced that would not be detected by the plaque assay used. It is possible that the required cellular enzyme is not present or needs to be induced or upregulated during activation of normal B cells. If enzymatic activity is low in infected B cells it is possible that few copies of F protein on the infected cell surface are in active form. Therefore, contact of an

infected B cell with the indicator monolayer may permit more efficient contact with a greater number of cleaved F protein molecules, thereby facilitating fusion and subsequent infectious center formation. Alternatively, released virion particles may contain a limited number of processed F glycoproteins which deters PFU detection. That B cells, in general, are not lacking in this enzyme is demonstrated by the observation that the murine myeloma, Sp2/0, and the hybridoma, 36-65, cell lines are permissive for NDV.

The question of incomplete F protein processing in virions produced by the B cell populations was analyzed directly by including trypsin, which cleaves F_o to an active form, in the media overlay mixture for the plaque assay (see methods). The data, shown in Table 11, demonstrate no differences in the number of PFUs assayed either with or without trypsin indicating that the lack of PFUs detected is not due to incomplete processing of the F glycoprotein in the B cell populations tested. The avirulent NDV strain B1 Hitchner, which requires cleavage of the F protein, was used as a control for this assay with less than 10 PFU/ml observed without trypsin and 1.7 X 10^6 PFU/ml with trypsin. These results indicate that the lack of PFU synthesis detected in NDV infected B cell

culture occurs at another level in NDV replication.

The lack of PFU synthesis by NDV infected B cells could be due to limits on infection at a number of levels, such as low level synthesis of a particular viral protein(s) (transcriptional or translational controls), defects in genomic replication, or virion assembly and maturation. To begin to study these possibilities, the progress of NDV infection was monitered by examination of viral protein synthesis. NDV viral proteins are observed in all populations of B cells, when total cell lysates labeled with ^{35}S-methionine were analyzed (Figure 12). Freshly isolated resting B lymphocytes (lane 2), unstimulated cultured B cells (lanes 4 and 14), splenic SN (lanes 6 and 16), anti-Ig (lanes 8 and 18), and LPS (lanes 10 and 20) activated B cells all support NDV viral protein synthesis. Lane 12 is from infected 36-65 cells which served as a control population of supportive cells. The prominant NDV viral proteins observed in total cell lysates are NP and P, which co-migrate, and M protein.

The synthesis of both the viral HN and L proteins can be detected by immunoprecipitation of infected cell lysates with rabbit anti-NDV serum (Figure 13 A, lanes 1, unstimulated; 2, unstimulated cultured; 4, splenic SN; 6, anti-Ig; 8, LPS; and 10, 36-65) or the HN protein alone by

using a monoclonal antibody (HN 2a, Figure 13 B, lanes 2, unstimulated; 4, unstimulated cultured; 6, splenic SN; 8, anti-Ig; 10, LPS; and 12, 36-65). The cleaved F protein co-migrates with NP and P in this gel system. In figure 13 A, the inability to detect a unique protein band migrating between the HN and the NP/P band, where F_o would migrate, in infected cell lysates compared to uninfected cells supports the notion that F protein cleavage is not a likely problem in these B cells. To confirm F protein synthesis and cleavage a monoclonal antibody to the NDV F protein, kindly donated by R. Iorio, will be used.

The ability to synthesize NDV viral proteins is consistent with the possibility that the infectious centers observed from infected B cell populations are the consequence of fusion between B cells and the indicator monolayer. The capacity of unstimulated and lymphokine stimulated B cells to synthesize NDV proteins but not VSV proteins (Figure 3) illustrates a level of contrast in B cell support of these two viruses. NDV protein synthesis by unstimulated B cells also helps to explain the higher background level of infectious center formation compared to VSV infection (Tables 4, 5, and 10). That all populations of B lymphocytes tested, even freshly isolated, apparently synthesize all NDV proteins without

PFU production suggests a block in virus replication at a point beyond primary translation. To further characterize B cell activation requirements for support of NDV infection, an assessment of lymphokine action on anti-Ig stimulated B cells was done.

Since stimulation of small resting B cells for 48 hours through the anti-Ig activation pathway appeared to be as effective as LPS for supporting NDV infection, I asked whether further differentiation along this activation pathway facilitated by lymphokines would produce a more supportive B cell population, as was the case for VSV infection. As shown in Table 12 (column -SN), activation for 48 hours with anti-Ig and lymphokines from a Th_2 T cell clone produced no significant increase in the number of infectious centers over anti-Ig or LPS stimulation alone. Furthermore, subsequent culture of infected B cells for an additional 24 hours with lymphokines had no further effect on NDV PFU synthesis in these populations (shown in Table 12, column +SN). These results demonstrate that the ability of anti-Ig activated B cell populations to support a productive NDV infection is not further enhanced with the additional differentiation induced by the action of lymphokines.

DISCUSSION

The experiments presented here demonstrate that, in contrast to VSV, freshly isolated unstimulated small B cells can support NDV infection, at least so as to produce infectious centers. This population begins to decline after 24 hours in culture. B cells activated from the resting G_o state into the G_1 phase of the cell cycle with lymphokines support, at best, a 2-fold increase in infectious centers compared to unstimulated cultured B cells. More likely, this number reflects lymphokine-mediated enhancement of B cell viability concurrent with a loss in viability of unstimulated B cells by 48 hours in culture rather than a direct lymphokine induced increase in B cell support of NDV. B cells stimulated into the S phase of the cell cycle with anti-Ig support an increase in number of NDV infectious centers approximately 5-fold over that observed in unstimulated B cell populations. B cells activated further along their differentiation pathway with anti-Ig and lymphokines or LPS support no further increases in numbers of infectious centers than that observed with activation by anti-Ig alone. All B cell populations, even unstimulated B cells, synthesize NDV viral proteins but fail to produce high levels of PFUs (Tables 10 and 12). The highest level of PFU synthesis is

detected in anti-Ig stimulated B cells, approximately 1 PFU per infectious center. Therefore, while the B cell activators used in these experiments support an increase in the number of B cells capable of supporting NDV protein synthesis, indicated by infectious center formation, no populations are produced which support a fully permissive NDV infection, defined here as high level PFU synthesis.

It is possible that the lack of PFUs detected from culture SN of NDV infected B cells is the result of adsorption of released virus particles by other cells in culture. Infected B cells were cultured overnight (for 24 hours post-infection) in 24 well tissue culture plates at a cell concentration of approximately 5 x 10^5/ml (total volume of 1 ml of media) for the analysis of PFU synthesis in these experiments. Although the ratio of infected : uninfected B cells would remain the same, lowering the cell concentration during the culture time may facilitate the detection of PFUs by reducing the overall density of the cultures. Adsorption of virus particles would be suggested by finding that 10-fold cell dilutions result in less than 10-fold reductions in virus titer. It is also possible that NDV PFU synthesis is delayed in infected B cell cultures. Therefore, PFU production will be assayed at 12 hour intervals up to three days post-infection in

order to assess the rate of PFU accumulation. Infected B cell cultures can also be set up under the same stimulating conditions used prior to infection to see if additional activation time enhances PFU synthesis.

NDV PFU production by B cells may also be limited by interferon effects. Interferon may be more effectively induced in B cells by NDV than VSV infection, subsequently causing low PFU production. Not only can viruses vary in their ability to induce interferon (Joklik, 1985), but the ability of particular host cells, in this case B lymphocytes in different activation states, to be induced to synthesize interferon can vary. Differences in interferon induction in B cells has been seen, e.g., splenic B cells, partially purified by methods which would retain macrophages, efficiently induce interferon as a result of stimulation with poly I:C but not LPS (R. Kamin, 1981). Whether interferon induction was due to stimulation of macrophages present in the cell populations used or was dependent on the B cell state of activation/ differentiation was not addressed in these experiments. To address the possibility that interferon is responsible for the low NDV PFU production in my system, anti-interferon antibodies, specific for alpha, beta, and gamma interferon, can be added to the infected B cell cultures

and SNs subsequently assayed for PFU production. Increased NDV PFU production by any or all infected B cell populations treated with antibodies over the level of PFUs in untreated B cell populations would suggest that interferon induction by NDV infection results in limited PFU synthesis.

The general characteristics of NDV infection in all primary B cell populations tested, viral protein synthesis with low level synthesis of NDV PFUs, are quite similar to the characteristics observed when anti-Ig stimulated B cells are infected by VSV. It is conceivable that all B cell populations tested are restricted in support of NDV replication at a point(s) beyond protein synthesis, such as protein maturation, virus replication, assembly or release of virus particles and the restriction may be dependent on the particular activation signals a B cell population received. Many of the possible limitations on virus replication previously discussed for VSV infection of anti-Ig stimulated B cells (chapter 3) may apply for NDV infection and are briefly outlined here.

Restriction on PFU synthesis in NDV infected B cells may be the result of inappropriate maturation of NDV proteins. While no evidence was seen for lack of synthesis or incomplete processing of the NDV F

glycoprotein by the B cell populations tested (Table 11), a direct examination has not been done. The use of a monoclonal antibody for immunoprecipitation will confirm synthesis as well as enzymatic processing of the F protein. Localization of the F protein to the cell surface for its inclusion into virions can be analyzed by cell surface staining or with specific immunoprecipitation of cell surface molecules. Oligomerization of the NDV HN glycoprotein is also required for inclusion into NDV virus particles (Schwalbe and Hightower, 1982). Both monomer and oligomer forms of HN have been shown to be expressed on the cell surface, but only the oligomer is found in virus particles (Morrison et.al., 1990). As a method for studying HN protein processing in B cells, I have available a number of monoclonal antibodies to the HN glycoprotein of NDV (kindly provided by Dr. R. Iorio). These antibodies have been used to study NDV-HN protein processing both at the level of virion maturation (i.e. the requirement for oligomerization, Morrison et.al., 1990) and for the acquisition of antigenic sites during protein transport and maturation within the cell (T. Morrison, personal communication). The use of these well characterized reagents provides a powerful tool to assess oligomerization, transport, and surface expression of HN

in B cell populations and should indicate if B cells restrict NDV infection at these levels.

Another level of restriction to NDV replication in B cells may occur at the synthesis of positive or negative genomic RNA strands. Synthesis of full length genomic RNA is dependent on the synthesis of sufficient nucleocapsid proteins to signal the RNA dependent RNA polymerase to synthesize full length copies of the genome rather than viral mRNAs (review: Kingsbury, 1990). If synthesis of either form is limited, low level PFU production could be observed. An examination of replicative forms of RNA in these B cell populations can be done to assess this possibility. To ask this question, a cDNA of the NDV P gene, kindly provided by T. Morrison, will be cloned into a bluescript vector containing promoters for T3 or T7 polymerases, allowing synthesis of probes specific for each sense of the genomic RNA. As NDV proteins are synthesized in all B cell populations restriction at viral mRNA synthesis is not a likely restriction point and I have decided not to analyze each NDV gene. The use of a single gene probe can be used to determine if NDV transcription, like VSV, is upregulated by lymphokines.) RNA will be extracted from NDV infected B cells at specific times post-infection and analyzed for the

presence of positive and negative-sense genomic RNA as well as for increases in the synthesis of negative-sense genomic RNA which are expected at later times of infection if viral replication is supported by B cells. B cells may also support the preferential synthesis of defective interfering genomic forms. RNA can be extracted from particles released into infected B cell culture SNs and assessed for synthesis of defective genomic forms by Northern analysis.

Limitation on NDV infection in the B cell populations tested could also be caused by defective or restricted assembly and maturation of virus particles. An electron microscopic study can be done to examine whether assembly of viral nucleocapsids occurs in the cell cytoplasm and to look for association of nucleocapsids with the cell membrane containing the viral glycoproteins. Inhibition of release or instability of released particles is another possibility for the lack of PFU detection from primary B cells. That instability is not a likely explanation for the lack of PFUs detected is demonstrated by the fact that the level of PFUs detected is constant when assayed either freshly from cell culture or after freezing of the SNs (data not shown). Inhibition of release may be suggested by the electron microscopic

studies if assembly of viral components within the B cell appears normal. It is also possible that the released virions contain insufficient amounts of particular viral proteins required for infectious virus particles. This possibility can be examined by analyzing the protein composition of ^{35}S-methionine labeled particles released into the culture SN from infected B cell cultures and permissive cells for differences in levels of viral proteins using SDS gel electrophoresis.

Each of the aforementioned possibilities could account for the lack of NDV PFU synthesis by the B cell populations tested. It is possible that the unique stimulus the B cells have experienced may impose a different restriction on the viral replicative process. Protein maturation, genomic replication, or maturation of virions, can be tested to determine where there is an impact on NDV infection.

An intriguing aspect of these experiments is the difference in the ability of primary B cells, activated to different points in their activation/ differentiation pathway, to support infection of NDV or VSV. Unstimulated or lymphokine activated B cells support NDV but not VSV protein synthesis. Anti-Ig stimulated B cells demonstrate the same level of support for the two viruses, i.e. viral

protein synthesis and an increase in number of infectious centers over background but only low level synthesis of PFUs. Anti-Ig and lymphokine stimulated cells which are fully permissive for VSV, supporting high levels of PFU synthesis, only support NDV protein synthesis and low PFU production. LPS activated cells, which are the most supportive for VSV both in the number of cells producing infectious centers as well as PFU synthesis, support NDV protein synthesis, no further increase in infectious center formation over that seen with anti-Ig stimulation and low level of NDV PFU synthesis. These results may suggest a requirement for different cellular factors by these two viruses for support of infection and that the appropriate factors for VSV but not for NDV are induced during B cell activation by the stimulators used.

The observed differences in level of PFU synthesis are due to an as yet undefined cellular function and are not apparently the consequence of the B cell populations supporting different levels of NDV and VSV viral protein synthesis. This is seen when aliquots taken from the same cell populations are infected with either NDV or VSV. Figure 14 demonstrates that when equivalent amounts of infected B cell lysates (also equivalent amounts of ^{35}S-methionine incorporation) are compared the level of

synthesis of the most prominant viral proteins, the NDV NP and P proteins or the VSV N and NS proteins, are similar. These comparable levels of viral protein synthesis are seen in populations of anti-Ig stimulated B cells which fail to produce either NDV or VSV PFUs (lanes aIg, N and V) and in B cell populations stimulated with anti-Ig and lymphokines which are fully permissive for VSV but not NDV (lanes aIg +SN, N and V). These results suggest that the failure of anti-Ig and lymphokine stimulated B cells to synthesize NDV PFUs is likely due to other host cell factors influencing the virus infections.

Experimental evidence for the idea that these two viruses utilize different cellular functions during their infective process is provided by a number of studies. The surface glycoproteins of these two virus families are expressed on opposite cell surfaces in polarized cells (Rodriquez-Boulan and Sabatini, 1978), and the rate of surface glycoprotein expression differs when the same cell type is infected with NDV or VSV (Morrison and Ward, 1984). That B cells can support a productive NDV infection is shown by the fact that the B cell hybridoma, 36-65, or the myeloma, SP2/0, support NDV to the same extent observed for NDV permissive chick embryo fibroblasts, producing up to 25 PFU per infectious center.

This suggests that either the differentiation state of these B cell lines or the transformation event which allowed continual growth in culture induced cellular changes required for high level NDV PFU synthesis. Alternatively, primary B cells may produce host cell factors which in some way directly inhibit NDV infection and the loss of expression in these permissive B cell lines results in enhanced susceptibility to NDV.

One class of host factors that have received a lot of interest are members of stress protein families. These proteins have been shown to fulfill some host cell functions required by a number of viruses to undergo an infective/replicative process, ex. the heavy chain binding protein (also called hsp 78, BiP, or GRP78) is important for oligomerization of VSV viral glycoprotein (de Silva et.al., 1990). Stress proteins are induced in cells by normal processes like B cell activation (Spector et.al., 1989; Wiest et.al., 1990) and by virus infection (Sarnow, 1989; Young, 1990; Collins and Hightower, 1982; Garry et.al., 1983). These proteins may have protective functions for cell survival or in turn may be utilized by the viruses to support infection (Young, 1990; Sarnow, 1990; de Silva et.al., 1990). That NDV and VSV may utilize different stress proteins is suggested by studies

that demonstrated that NDV infection of chick embryo cells induced the synthesis of hsp20, hsp30, hsp70, hsp78, and hsp90 proteins (Collins and Hightower, 1982) whereas VSV infection of the same cell type induced hsp70 and hsp90 proteins (Garry et.al., 1983). Therefore, it is possible that the B cell activation regimins used induce the synthesis of stress proteins which support VSV infection while either inducing other proteins that directly inhibit NDV or block the ability of NDV to induce required host cell proteins. Studies that examine the overall expression of these stress protein families in B cells at different activation states or induced by different activators may provide insights into host cell factors that influence virus infection of B lymphocytes.

Table and Figure Legends

Table 9

B cell enriched populations were cultured with the indicated stimulus for 24, 48, or 72 hours prior to NDV infection. Serial dilutions of infected cells were plated onto chick embryo fibroblast monolayers, overlayed with 2% agarose/agar media and plaques counted at 2 and 3 days. As controls the myeloma Sp2/0 produced 452 and 420 infectious center per 10^3 cells plated at 24 and 48 hour time points, respectively, and CHO cells produced 370 infectious centers per 10^3 cells plated in the 72 hour experiment.

a number of infectious centers per 10^3 cells plated, mean of three serial dilutions

b not done

Table 10

Small elutriated B cells were cultured with the indicated stimulus for 24 or 48 hours prior to infection with NDV. In experiment A freshly isolated B cells were also assayed for support of NDV. The hybridoma, 36-65, was used as a control in these experiments. All values are per 10^3 cells assayed. In experiment A 36-65 produced

322 I.C. and 4,830 PFU at 24 hours and 295 I.C. and 6,200 PFU at 48 hours. In experiment B 36-65 produced 270 I.C. and 3,900 PFU at 24 hours and 339 I.C. and 7,800 PFU at 48 hours.

a number of infectious center per 10^3 cells plated, mean of three serial dilutions

b number of PFU per 10^3 cells cultured, mean of two serial dilutions

Table 11

Small elutriated B cells were cultured for 48 hours with the indicated stimulus prior to infection. Culture SNs were collected 24 hours post-infection from infected B cell cultures and were assayed for PFU production in the presence or absence of trypsin. As control for trypsin activity the NDV avirulent strain B1 Hitchner gave <10 PFU/ml without trypsin and 1.7×10^6 PFU/ml with trypsin.

a number of PFU per 10^3 cells cultured, mean of three serial dilutions

Table 12

Small elutriated B cells were culture with the indicated stimulus for 48 hours prior to NDV infection.

Infected B cells were cultured for 24 hours post-infection in the presence or absence of Th_2 T-cell derived lymphokines (final concentration 50%). As control 36-65 produced 6,470 PFU per 10^3 cells cultured.

a number of infectious center per 10^3 cells plated, mean of three serial dilutions

b number of PFU per 10^3 cells culture, mean of three serial dilutions

Figure 12

Small elutriated B cells were infected with NDV on the day of isolation (lanes 1 and 2), cultured 24 (lanes 3-10), or 48 hours (lanes 13-20) with the indicated stimulus prior to infection. Cells were labeled with ^{35}S-methionine in media for 2 hours between 8-10 hours post-infection, lysed, and equivalent cpm were analyzed on a 10% SDS acrylamide gel. All samples are shown as paired lanes uninfected (odd number lanes) and infected (even numbers). The hybridoma, 36-65, (lanes 11 and 12) was used as control in each experiment.

Figure 13

Immunoprecipitation with rabbit anti-NDV serum (A) or monoclonal anti-NDV HN 2a (B) were carried out on ^{35}S-

methionine labeled uninfected and infected B cell lysates. The samples of B cells used were infected after 48 hours of stimulation except for those in panel A, lane 1 and panel B, lanes 1 and 2 which were samples of freshly isolated B cells.

Panel A: Samples from B cells in lane 1, unstimulated freshly isolated and lane 2, unstimulated cultured or B cells stimulated with lanes 3 and 4, splenic SN; lanes 5 and 6, anti-Ig; lanes 7 and 8, LPS. Lanes 9 and 10 are lysates from the hybridoma 36-65. Lanes 3, 5, 7, and 9 are uninfected cell lysates and lanes 1, 2, 4, 6, 8, and 10 are infected cell lysates.

Panel B: Samples from B cells in lanes 1 and 2, freshly isolated; lanes 3 and 4, unstimulated cultured; lanes 5 and 6, splenic SN; lanes 7 and 8, anti-Ig; lanes 9 and 10, LPS; and lanes 11 and 12, 36-65. Lanes 1, 3, 5, 7, 9, and 11 are uninfected cell lysates and lanes 2, 4, 6, 8, 10, and 12 are infected cell lysates.

Figure 14

Small elutriated B cells were cultured for 48 hours with the indicated stimulus prior to infection and then ^{35}S-methionine labeled for 2 hours between 8 and 10 hours post-infection. Samples from each stimulated

population were taken for uninfected (U), NDV infected (N), and VSV infected (V). Equivalent cpm (also equivalent cell lysates for each stimulus) were loaded for comparison on a 10% SDS acrylamide gel.

TABLE 9

INFECTIOUS CENTER ASSAY OF B CELL ENRICHED SPLEEN CELL POPULATIONS INFECTED WITH NDV AT VARIOUS TIMES AFTER IN VITRO CULTURE

	INFECTIOUS CENTERS[a] (per 10^3 cells plated)		
STIMULUS	24 hr.	48 hr.	72 hr.
NONE	31	59	nd[b]
SPLENIC SN	58	106	53
ANTI-Ig	56	236	236
ANTI-Ig + SN	66	347	230
LPS	40	125	121

TABLE 10

SMALL RESTING B CELLS CAN BE ACTIVATED TO PRODUCE INCREASED NUMBERS OF NDV INFECTIOUS CENTERS WITHOUT SIGNIFICANT PFU PRODUCTION

STIMULUS	INFECTIOUS CENTERS[a] (per 10^3 cells plated)		PFU[b] (per 10^3 cells cultured)	
A				
FRESHLY ISOLATED	27		0	
	24 hr.	48 hr.	24 hr.	48 hr.
NONE	52	38	1	4
SPLENIC SN	36	86	1	4
ANTI-Ig	39	210	2	85
LPS	80	140	1	3
B				
NONE	40	22	2	7
Th1 SN (D1.6)	40	39	1	7
Th2 SN (CDC 35)	39	39	10	18
ANTI-Ig	31	66	12	82
LPS	37	84	4	12

TABLE 11

F PROTEIN CLEAVAGE DEFECTS IN B CELLS
DO NOT ACCOUNT FOR LOW PFU PRODUCTION

STIMULUS	PFU [a] (per 10^3 cells cultured) -trypsin	+trypsin
NONE	6	4
Th2 SN	11	17
ANTI-Ig	130	147
ANTI-Ig + SN	81	94
LPS	9	11

TABLE 12

NDV PFU PRODUCTION BY INFECTED ANTI-Ig STIMULATED B CELLS IS NOT ENHANCED BY CO-CULTURE WITH LYMPHOKINES

	INFECTIOUS CENTERS [a] (per 10^3 cells plated)	PFU [b] (per 10^3 cells cultured)	
STIMULUS		-SN	+SN
NONE	9	6	9
Th2 SN	18	11	15
ANTI-Ig	56	130	165
ANTI-Ig + SN	60	81	116
LPS	65	9	14

FIGURE 12

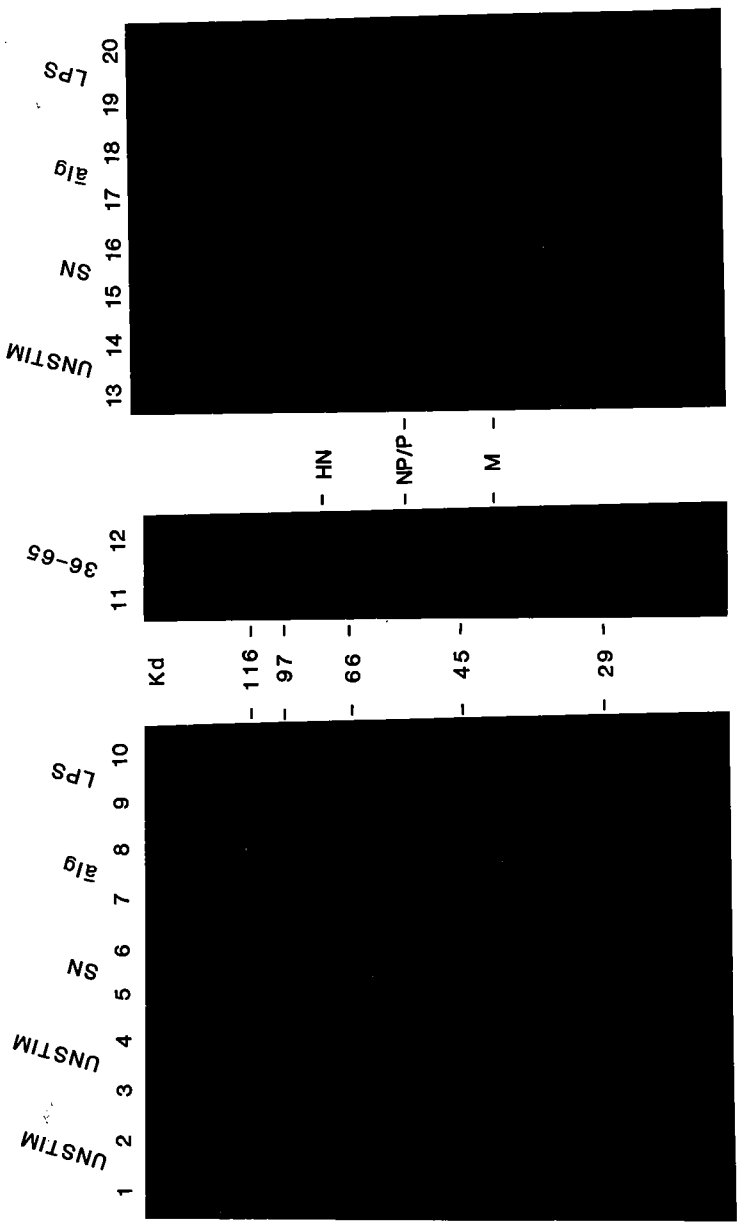

UNSTIM
SN
αIg
LPS
UNSTIM
SN
αIg
LPS
36-65
Kd
116
97
66
45
29
HN
NP/P
M

FIGURE 13

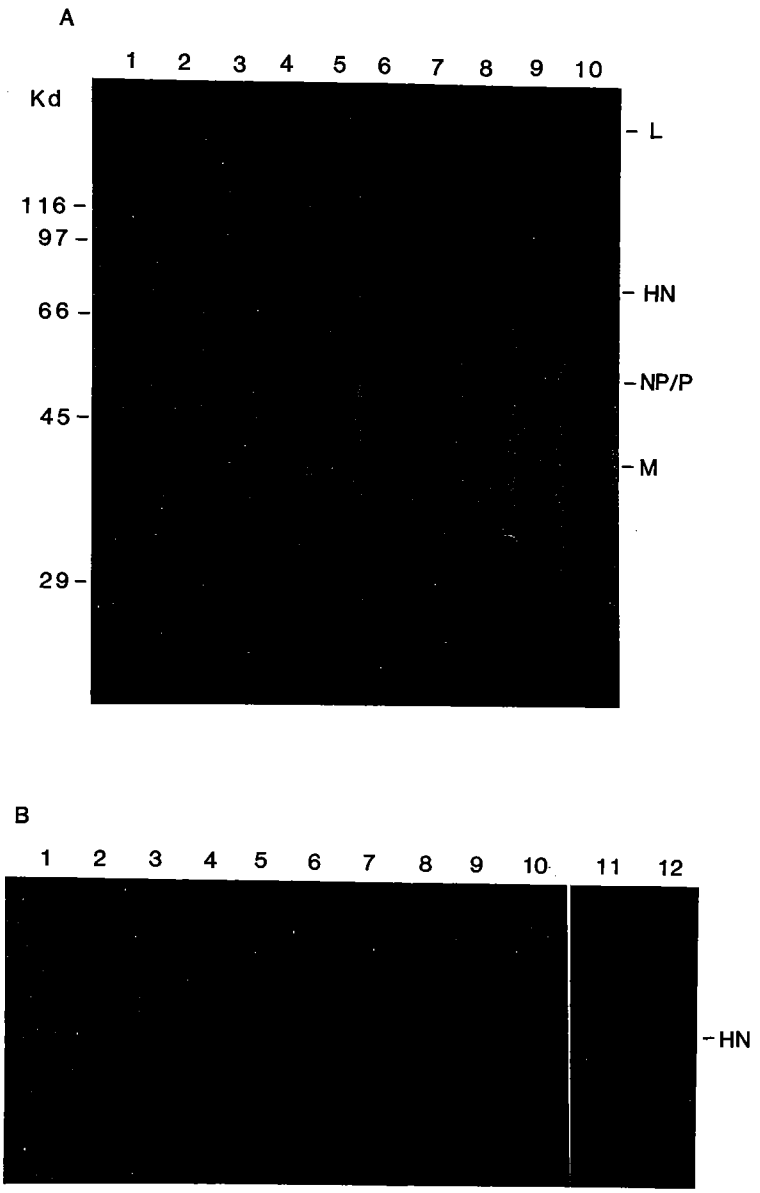

A
1 2 3 4 5 6 7 8 9 10
Kd
116
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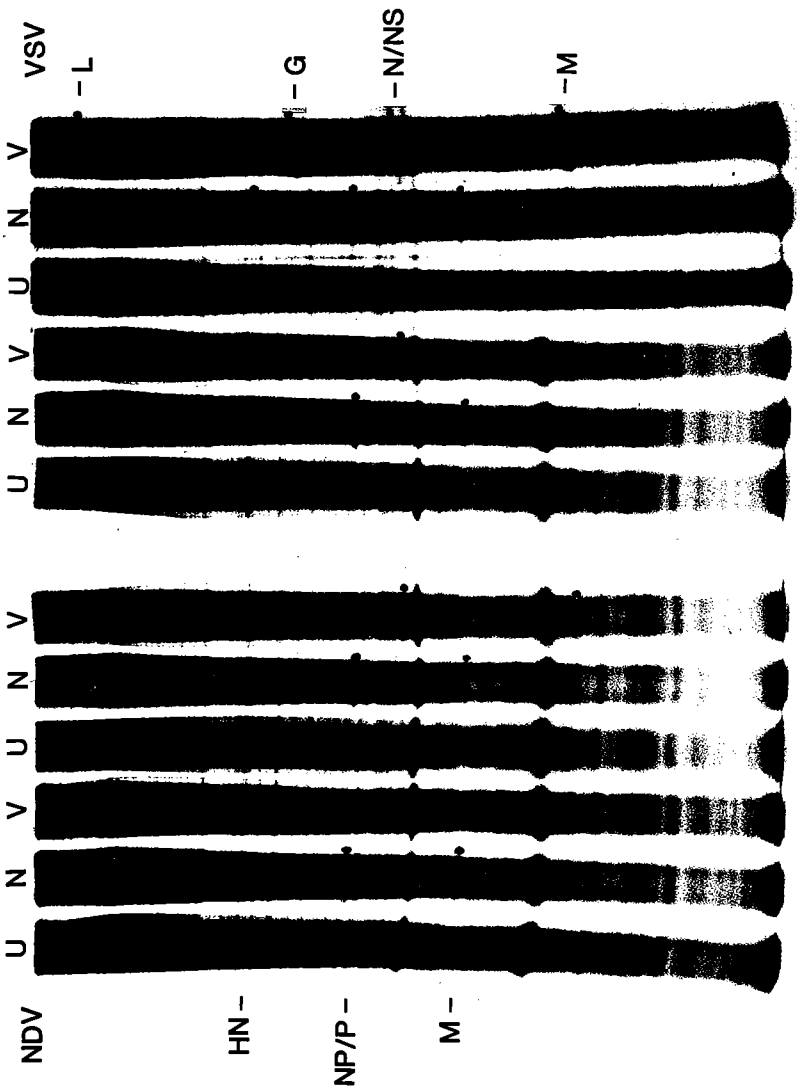
FIGURE 14



CHAPTER 5

DISCUSSION

In this dissertation, I describe an experimental system which provides for an analysis of the ability of primary B lymphocytes in different activation states or exposed to different activators to function as host cells for virus infection. Because transformed B cell lines representing different stages of B cell differentiation exhibited levels of virus support not seen in primary B cell populations at equivalent differentiation stages, primary B cells were considered the most appropriate model for analysis. Because the general requirement for lymphocyte "activation" for viral PFU production has been demonstrated by other investigators besides myself, unique aspects of the association between activation and support of virus infection are reported here. The progress of VSV infection in primary B lymphocytes is directly dependent on their activation state. B cell control on VSV infection at the translational level was observed in unstimulated B cells and in lymphokine stimulated B cells, this latter observation despite the demonstration of lymphokine mediated enhancement of viral mRNA transcription. B cell entry into the S phase of the cell cycle by anti-Ig stimulation released the translational

block but still restricted VSV infection as high level PFU synthesis required B cell stimulation with LPS or co-stimulation with anti-Ig and lymphokines. Somewhat surprisingly, none of the B cell stimulators used induced full support for NDV, when assessed by high level PFU synthesis, although all populations supported NDV protein synthesis. These results suggest that complex questions of translational control and transcriptional regulation may be addressed in primary cell populations that exist in functionally distinct cohorts as a consequence of the activation regimen to which they have been subjected.

The results of these experiments also suggest that these two closely related viruses may be dependent on different cellular factors which are induced for VSV but not NDV by the B cell activators used or that the process of infection of B cells by these two viruses induces different cellular responses, e.g. NDV but not VSV may be a strong inducer of interferon in B cells (R. Kamin, personal communication). Whatever the mechanism, the system discussed here predictably activates primary B cells allowing the further characterization of the process of infection and host cell factors required by these and other viruses.

This system also provides a new bioassay for B

cell lymphokines. Previously, the activity of lymphokines or other B cell activators have been assessed by their ability to support B cell proliferation and/or antibody secretion or the induction of antibody isotype switching. The modulation of virus expression following activation and/or lymphokine exposure is a new analytical approach for the assessment of B cell biochemical capabilities that are induced as a consequence of different B cell activators. The finding that anti-Ig or LPS activated B cell populations have very different responses to the same populations of lymphokines when assayed for the ability to support VSV infection illustrates the potential for detecting previously unknown lymphokine effects on B cells and the biochemical changes that underlie those effects. The contrasting effects of lymphokines observed with anti-Ig or LPS support the notion that lymphokine receptor expression and response characteristics are dependent on the signalling pathway used to trigger B cell activation/ differentiation to antibody secretion or that the subpopulation of B cells which responds to particular activation signals, anti-Ig vs. LPS, have intrinsic differences in lymphokine responsiveness. Further experiments using defined, recombinant lymphokines in combination with anti-Ig or LPS stimulation can be done to

assess the molecular/biochemical consequences of the expression of particular lymphokine receptors on these populations of primary B cells.

The analysis of B cell support for VSV infection can also be used with stimulators that allow for further dissection of the anti-Ig activation pathway. B cell activation with both phorbol esters and calcium ionophores causes phosphatidylinositol breakdown and calcium mobilization, mimicking the responses of anti-Ig stimulation in normal B cells, and allows B cell entry into the S phase of the cell cycle (Klaus et.al., 1986; Gold et.al., 1990). Activation with either of these stimulators alone is not sufficient to induce DNA synthesis but provides the opportunity to examine whether either phorbol ester and/or calcium ionophore stimulation alone will release the VSV translational block. Second, because anti-Ig and lymphokine stimulation produce B cells fully permissive for VSV, will phorbol esters and/or calcium ionophores serve the same function when used in combination with lymphokines? Along this same line of investigation, other B cell activators which induce the same initial changes as anti-Ig in B cells can also be used. Conconavalin A induces B cell entry into the G_1 stage of the cell cycle (Hawrylowicz and Klaus, 1984) and

antibody to Lyb-2, a B cell differentiation antigen that is involved in B cell activation, causes B cell entry into G_1 and increased surface Ia expression (Subbarao et.al., 1988). Will these inducers support the translation of VSV mRNAs or upon co-stimulation with lymphokines will they enhance VSV PFU production? The examination of support for VSV infection may demonstrate biochemical differences in B cells activated with these stimulators, either alone or with lymphokines, and provide further information on the signalling events required for B cell activation and antibody secretion.

Additionally, studies of the hetergeneity of B cell responsiveness to lymphokines and other activators, monitered by their ability to support VSV infection, can be extended by examining other populations of B cells. B cells in the CBA/N mouse, which carries the xid defect (X-chromosome-linked immunodeficiecy) have been used as a model to assess the function of a distinct B cell subpopulation (Scher, 1982). As a consequence of genetic differences in x-linked genes which influence B cell differentiation, xid B cell responses to activation signals differ from normal B cells (Cohen et.al., 1985; Siegel et.al., 1985; Ono et.al., 1983; Sprent et.al.; 1985; Sprent and Bruce, 1984). Comparing the biological

properties and ability to support VSV infection by xid and normal B cell populations may provide further insights into the process of B cell activation. For example, will lymphokines stimulate xid B cells to support increased VSV mRNA transcription? Anti-Ig stimulation of xid B cells induces cell blastogenesis, inositol phospholipid breakdown and calcium mobilization that is observed with anti-Ig stimulation of normal B cells but fails to induce xid B cell entry into the S phase of the cell cycle (Rigley et.al., 1989; Sieckman et.al., 1978; Hawrylowicz, 1984). Therefore, does anti-Ig stimulation of xid B cells release the translational block in VSV infection? Unlike anti-Ig, phorbol esters and calcium ionophores stimulate DNA synthesis in xid B cells, like normal B cells (Hawrylowicz et.al., 1984; Klaus et.al., 1986; Rigley et.al., 1989), supporting the idea that anti-Ig activation signals for xid B cells differ at the level of signal transduction from normal B cells (Hawrylowicz et.al., 1984; Klaus et.al., 1986; Rigley et.al., 1989). An analysis of support for VSV infection in xid B cell populations stimulated with phorbol esters and/or calcium ionophores may further dissect the activation signal(s) required for release of the B cell block on VSV mRNA translation.

Stimulation of xid B cells with anti-Ig and lymphokines does not induce antibody secretion as in normal B cells, once again providing an opportunity for examining the B cell activation signals required for increased VSV PFU production. Whether co-stimulation of xid B cells with LPS and lymphokines will have the same suppressive effect on VSV PFU production as seen with normal B cell populations is another potential area of investigation. These studies on VSV infection of xid B cells may permit the dissection of B cell activation signals and provide insights into lymphokine and activation mediated regulation of B cells that may be associated with X-linked immunoregulatory elements.

Ly 1^+ B cells, a bona fide B cell subpopulation, are of increasing interest because they spontaneously secrete antibody, including autoantibodies, and are the predominant phenotype of spontaneous B cell lymphomas and leukemias (Braun et.al., 1986; Hardy and Hayakawa, 1986; Herzenberg et.al., 1986), can also be assessed for their ability to support VSV infection. This functionally distinct population of B cells is primarily found in the peritoneal cavity and, unlike normal B cells, is capable of self-renewal from Ig^+ precursors and may also be undergoing cell cycling. These characteristics suggest

that Ly 1^+ B cells exist in a different activation state with different biochemical capabilities than splenic B cells. Because Ly 1^+ B cells are capable of self renewal, will they support VSV infection without additional stimulation? Since Ly 1^+ B cells may be cycling, will lymphokine stimulation alone result in a fully permissive cell population for VSV or permit translation of VSV proteins? While normal B cells require both phorbol esters and calcium ionophore stimulation to enter S phase of the cell cycle, stimulation with phorbol esters alone is sufficient for Ly 1^+ B cells (Rothstein and Kolber, 1988). Will phorbol ester activated Ly 1^+ B cells, which have entered S of the cell cycle, permit synthesis of VSV proteins? Lymphokines have also been shown to affect Ly 1^+ B cell responses, e.g. IL-4 enhances peritoneal B cell stimulation induced by phorbol esters (Rothstein, 1989). Therefore, what effect will lymphokines have on VSV support by phorbol ester activated Ly 1^+ B cells? An examination of the ability of Ly 1^+ B cells to support virus infection, with or without activation or lymphokines, provides another means for assessing the biochemical capabilities and regulation of this distinct B cell subpopulation that is associated with lymphomas and autoimmune disease.

Finally, this system of B cell activation can be used to assess the B cell support for other viruses which directly infect mice and use B lymphocytes as hosts. This system can also be extended to viruses which require a receptor that is not expressed on murine cells by using transgenic mouse models to change virus tropisms. For example, poliovirus has been demonstrated to infect human lymphocytes, and virus replication is enhanced by lymphocyte activation (Okada et.al., 1987; Willems et.al., 1969). The poliovirus receptor has been cloned, and a transgenic mouse has been made for study (Mendelsohn et.al., 1989; M. Chow, personal communication. The use of primary B cells rather than transformed cell lines provides a model for the study of inducible biochemical changes effecting host cell/virus requirements for infection.

In summary, this experimental system provides the opportunity to study primary B lymphocytes as host cells for viruses as well as being able to study activation induced changes in the biochemistry of B cells by assaying support for virus infection.

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