Université de Montréal

Regulation of Cytokine Gene Expression by the Epstein-Barr Virus and its Envelope Glycoprotein gp350.

par

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Thèse présentée à la Faculté des études supérieures en vue de l'obtention du grade de Philosophiae Doctor (Ph.D.)

FEVRIER 2000

Université de Montréal

Faculté des études supérieurs

Cette thèse intitulée:

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présentée par:

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Thèse acceptée, Fevrier 2000.

SUMMARY:

Epstein-Barr virus (EBV) is a member of the herpesvirus family that has potent immunomodulatory and oncogenic effects. Following the initial infection of B-lymphocytes, EBV establishes a period of latency that can be interrupted with viral reactivation and propagation which occurs during host immunosuppression or immunodeficiency.

To better understand the interaction of EBV with immune cells, we initiated a series of experiments using monocyte/macrophages to study the production of pro-inflammatory cytokines (interleukin-1beta [IL-1β], interleukin-6 [IL-6] and tumor necrosis factor-alpha [TNF-α]). Our work examined the interaction of EBV and its major envelope glycoprotein (gp350) with these cells. Monocytic cells secrete a multitude of immunoregulatory agents (cytokines) and IL-1β, IL-6 and TNF-α are among the most critical in regulating normal immune activity.

Our previous results found that infectious EBV suppressed the production of TNF-α and IL-1β while not affecting the production of IL-6. During my doctoral work, I chose to more closely study the intracellular mechanisms regulating the production of these cytokines in monocytic cells treated with EBV-gp350. In addition, I focused on the interaction of EBV-gp350 with monocytes and the role played by second messenger systems following its interaction with cell surface CD21/CR2.

Our results demonstrated that EBV-gp350 interacting with CD21/CR2 was able to stimulate the production of IL-1β, IL-6 and TNF-α RNA and protein. This increase was demonstrated in purified adherent monocyte/macrophages and in an established monocytic cell line (U937). The results may be partially explained by data showing that EBV-gp350 increased the RNA half-life of both TNF-α and IL-6. In addition, we examined the involvement of several cellular transcription factors and found that NF-κB

was involved in the transcriptional activation of all three cytokine genes. NF-κB is a family of transcription factors that regulate a number of cellular and viral genes; this protein family remains inactive in the cytoplasm but following cellular stimulation migrates to the nucleus to bind a specific decameric DNA sequence. Our results also show that while EBV-gp350 acts through CD21/CR2 to specifically stimulate the protein-kinase C and phosphatidylinositol-3-kinase signal transduction pathways; EBV activation of IL-6 requires multiple pathways for gene activation.

In conclusion, our results clearly demonstrate that EBV-gp350 acts through CD21/CR2 on monocytic cells to augment the production of cytokines IL-1β, IL-6 and TNF-α. We go on to demonstrate the direct activation of NF-κB proteins through specific signal transduction pathways

CHAPTER ONE:

To my wife Vana, my family

and Dr. J. Menezes for their

astronomical patience

and support.

PREFACE:

A doctoral thesis is obtained following the discovery of unique results that advance knowledge in a particular field. Through logical systematic independent reasoning, the candidate acquires the ability to observe results and progress scientific work to its next level. This is the basis of an independent scientist.

I chose to continue the work initiated by Dr. J. Gosselin, due to my initial involvement in that particular project. Since my M.Sc. degree I have always been fascinated by the interaction of viruses and the immune system. Through my initial work with HIV, I acquired knowledge invaluable in my Ph.D. training.

Through 6 years of part-time study, sufficient data was generated to prepare the following manuscripts:

1. **D'Addario, M.,** A. Ahmad, J. Xu, and J. Menezes. 1999. Epstein-Barr virus envelope glycoprotein gp350 induces NF-κB activation and IL-1β synthesis in human monocyte-macrophages involving PKC and PI3-K. **FASEB J.** 13: 2203-2213

2. Binding of the Epstein-Barr virus major envelope glycoprotein gp350 results in the upregulation of TNF-α gene expression in monocytic cells via NF-κB involving PKC, PI3-K and tyrosine kinases. **M. D'Addario**, A. Ahmad, and A.J. Morgan, and J. Menezes (submitted to Journal of Molecular Biology).

3. Epstein-Barr virus and its glycoprotein upregulate IL-6 in human B-lymphocytes via CD21, involving activation of NF-κB and different signal transduction pathways. **D'Addario, M.,** T. A. Libermann, A. Ahmad, and J. Menezes (submitted to Journal of Immunology).

I have also contributed to the following manuscripts generated in our laboratory:

1. Ngola, B.B., J. Menezes., **M. D'Addario**, J. Xu, J. Ongradi and A. Ahmad.1999. Effect of human herpes virus-7 (HHV-7) on the modulation of cytokine synthesis and cell proliferation in peripheral blood mononuclear cells. *J. Leuk. Biol.* 66: 822-828.

2. Xu, J., A. Ahmad, M. Blagdon, **M. D'Addario,** J.F. Jones, R. Dolcetti, U. Parsad, and J. Menezes. 1999. The Epstein-Barr virus (EBV) major envelope gp350/220 specific antibody reactivities in the sera of patients with different EBV-associated diseases. *Int. J. Cancer* 79: 481-486.

3. Xu, J, A. Ahmad, **M. D'Addario**, L. Knafo, J.F. Jones, R. Dolcetti, U. Parsad, E. Vaccher and J. Menezes. 2000. Analysis and significance of anti-latent membrane protein-1 antibodies in the sera of patients with EBV-associated diseases. *J. Immunol.* 164: 2815-2822.

Résumé

La famille des virus *Herpès* compte huit membres qui infectent l'homme et causent des pathologies sérieuses. Ces virus peuvent infecter les différentes cellules immunocompétentes et causer des dysfonctionnements du système immunitaire. Ces défauts fonctionnels peuvent être dus à une simple immunomodulation ou à des effets immunosuppressifs sérieux donnant lieu à des états d'immunodéficience. Un des virus les plus immunotropiques de cette famille est le virus d'Epstein-Barr (EBV). L'EBV est un puissant agent immunomodulateur qui peut influencer la synthèse de nombreuses cytokines et ces dernières jouent un rôle important dans l'immunorégulation.

Le principal but de mon travail était d'étudier la régulation de la synthèse des cytokines suite à l'interaction d'EBV avec les cellules monocytaires du sang périphérique humain. Dans ce contexte, j'ai d'abord analysé (a) l'expression des gènes pour le facteur de nécrose de tumeurs (TNF) et l'interleukine-1 (IL-1) suite à cette interaction, et ensuite (b) l'implication des facteurs de transcriptions NF-κB dans cette expression. Mon hypothèse de travail était que la glycoprotéine majeure de son enveloppe, gp350, médie les effets régulateurs d'EBV au niveau de l'expression des gènes des cytokines et que ces effets comportent l'activation du facteur de transcription NF-κB. Les cellules monocytaires sont très importantes dans le déclenchement du processus inflammatoire et l'initiation de la réponse immunitaire (y compris pour l'activation des lymphocytes). Durant ce processus, il y a la production d'importants médiateurs / cytokines, en particulier IL-1, TNF et l'interleukine-6 (IL-6).

Nos études antérieures ont démontré que la production de cytokines TNF, IL-1 et IL-6 est fortement déréglée suite à l'interaction de cellules monocytaires avec le EBV. Nos résultats démontrent que la synthèse de ces cytokines après une infection virale varie considérablement, et ce dépendant

de la cytokine impliquée. On a aussi trouvé que l'EBV cause une diminution de la synthèse de TNF et IL-1, et une augmentation d'IL-6 tant au niveau de son ARN messager que de la protéine. Il est intéressant de noter ici que le virus inactivé aux rayons ultraviolets ou à la chaleur a des effets opposés au virus infectieux sur la synthèse de TNF et IL-1. J'ai ensuite analysé les effets de l'interaction de la gp350 avec son récepteur cellulaire CD21/CR2. Cette analyse avait comme but l'étude de l'effet de gp350, seule, sur la modulation de l'expression de ces cytokines.

Les résultats obtenus indiquent que l'activation de l'expression des gènes de TNF et IL-1 par gp350 est accompagnée par une augmentation des protéines NF-κB via l'activité enzymatique de la protéine kinase-C et phosphotidyl-inositol-3-kinase. Nos résultats indiquent aussi que la gp350 n'a pas d'effet activateur sur l'adenylate cyclase, phospholipase-C, protéine-tyrosine kinase, ou protéine kinase dépendante de Ca^{++}.

Nos résultats illustrent comment un virus comme l'EBV peut dérégler le système immunitaire en modulant la synthèse des cytokines immunorégulatrices. En outre, ces résultats démontrent que la gp350 d'EBV, toute seule, est capable de moduler la production de ces cytokines via l'activation des voies enzymatiques telles la protein kinase-C et phosphotidyl-inositol-3-kinase qui à leur tour activent le NF-κB.

En somme, les résultats obtenus indiquent que l'EBV et la glycoprotéine majeure de son enveloppe gp350 peuvent influencer considérablement les fonctions du système immunitaire, principalement via la modulation de la synthèse des cytokines. Ces effets modulateurs pourront jouer un rôle important aussi bien dans la réponse immunitaire de l'hôte aux infections que dans la pathogenèse de ces infections.

LIST OF ABBREVIATIONS:

ACT D:	Actinomycin-D
AP-1:	Activator protein-1
BCRF1:	EBV gene coding for viral interleukin-10
BL:	Burkitt's Lymphoma
cAMP:	Cyclic adenosine monophosphate
CD21:	Cluster of differentiation (type 21)
CHX:	Cycloheximide
CMV:	Cytomegalovirus
CREB:	cyclic AMP responsive element binding protein
CR2:	Complement receptor type 2
EBNA:	EBV nuclear antigen
EBV:	Epstein-Barr virus
EBERs:	EBV small non-polyadenylated small RNA
GM-CSF:	Granulocyte macrophage-colony stimulating factor
gp350:	glycoprotein-350
HD:	Hodgkins disease
HHV-6:	Human herpesvirus type-6
HIV:	Human immunodeficiency virus
HSV:	Herpes simplex virus
ICAM:	Intracellular adhesion molecule
IFN:	Interferon (alpha, beta and gamma)
Ig:	Immunoglobulin
I-κB:	Inhibitor of nuclear factor kappa B
IL-s:	Interleukins (-1α, -1β, -2, -6, -10, -15)
kbp:	kilo base pair
kDa:	kilo Dalton
LCL:	Lymphoblastoid cell lines
LMP:	Latent membrane protein
LPS:	Lipopolysaccharide
mAb:	Monoclonal antibody
MHC:	Major histocompatibility complex
NF-κB:	Nuclear factor kappa B
NPC:	Nasopharyngeal carcinoma
PBMC:	Peripheral blood mononuclear cells
PKC:	Protein kinase C
PI-3-k:	Phosphatidyl-inositol-3-kinase
PMA:	Phorbol myristate acetate
PTK:	Protein tyrosine kinase
SV40:	Simian virus-40
TGF:	Transforming growth factor (-α, -β)
TPA:	Tumor promoting antigen
UTR:	Untranslated region

1.0 INTRODUCTION

1.1 HERPESVIRUSES.

Herpesviruses are a family of viruses that infect a diverse range of hosts; at least eight of which are known to infect humans. All the viruses in this family have the capacity to infect and remain latent in their host indefinitely. Consisting of *Alphaherpesviridae*, *Betaherpesviridae* and *Gammaherpesviridae*, this virus family is known to be associated with many different pathologic states. Below is a brief description of each subset of herpesviruses and their associated diseases with respect to human infection.

The *Alphaherpesviridae* consist of herpes simplex virus-1 (HSV-1) and -2 (HSV-2), Simian herpes virus (SHV) and Varicella-Zoster virus (VZV). This class of herpes virus is capable of establishing lifelong latency in cerebral or spinal ganglia but may replicate quickly, spread rapidly and be cytolytic. Depending upon the immune status of the host and the site of infection, this virus family often produces transient pathologies. Infections by this class of herpesvirus usually occur in children and induce diseases prior to establishment of viral latency. Infections by HSV's usually result in oropharyngeal infections, genital herpes, keratoconjunctivitis, skin infections, encephalitis and possibly fatal neonatal herpes. Varicella Zoster virus infection usually produces chickenpox (Varicella) and zoster in older individuals.

The *Betaherpesviridae* class includes cytomegalovirus (CMV) and human herpesvirus-6 (HHV-6). CMV will most often infect children and neonates as they pass the birth canal. While most of the associated diseases are transient and uneventful, infections of the fetus may produce serious teratogenic effects including encephalitis, microcephaly, mental retardation, hepato-splenomegaly, chorioretinitis and inguinal hernias. Infections of older individuals will usually produce transient episodes of mononucleosis, pneumonitis, retinitis and/or gastrointestinal diseases. CMV and HHV-6 will often remain latent in lymphocytes, macrophages, salivary gland tissue and enterocytes and grow slowly.

The *Gammaherpesviridae* consist of Epstein-Barr virus (EBV) and human

herpesvirus-8 (HHV-8). While EBV is known to infect several different cell types, HHV-8 infection still remains mysterious as this virus has only recently been discovered on the basis of its association with Castleman's disease and the HIV-1 associated tumor Kaposi's sarcoma.

1.2. Introduction to Epstein-Barr Virus.

Epstein-Barr virus (EBV) is a member of the herpes virus family; first discovered in 1950, this virus is associated with several human tumors and is known to be antigenically distinct from other members of the herpesvirus family. EBV, lymphocryptovirus and rhadinovirus comprise the gamma herpesvirus subgroup (Kieff, 1996). While lymphocryptovirus and rhadinovirus primarily infect non-human primates, EBV is the only member of this subfamily to infect up to 95% of the human population. This subfamily of herpesvirus was initially established due to similarities in biologic properties, principally their ability to establish long periods of latency that can be interupted by periods of active viral proliferation.

Although initially discovered in African childhood tumors, EBV is present in all human populations and is associated with a number of disorders. EBV is associated with nasopharyngeal carcinoma (NPC), Burkitt's lymphoma (BL), Hodgkin's disease (HD) and certain T cell lymphomas (Alero Thomas et al., 1991, Neidobitek and Young, 1994 and Kieff, 1996). In its most common form, EBV establishes asymptomatic mucosal epithelial infections in the first 3 years of life (Henle et al., 1970). Infections in later life usually occur through salivary transfer of virus carried from epithelial cells (Kieff, 1996). EBV can also be isolated from oral squamous cells and has been associated with a number of oral diseases (Greenspan et al., 1985, Young et al., 1988 and Walling et al., 1994).

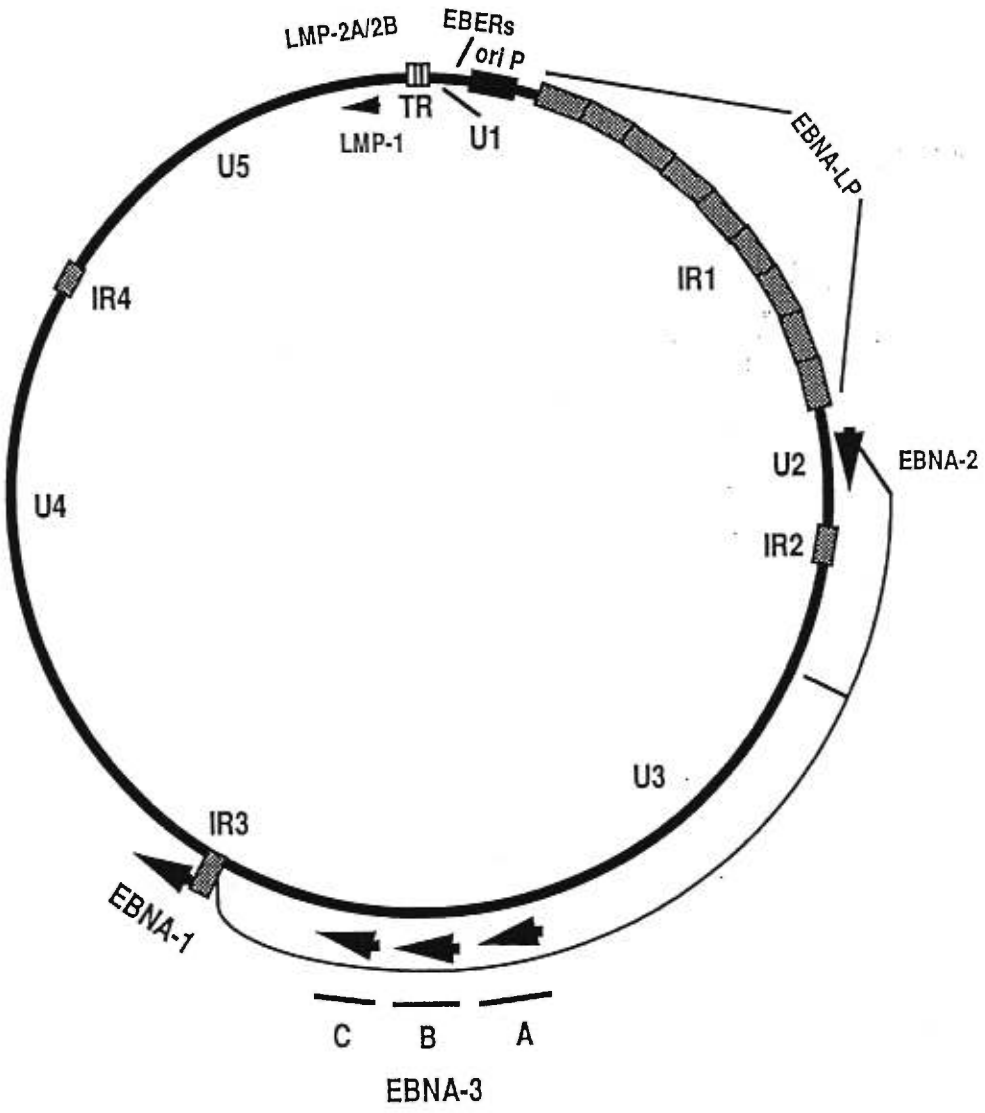
Almost all herpesviruses have a characteristic morphology; a toroid shaped core encircles the double stranded DNA, surrounded by a nucleocapsid containing 162 capsomere proteins (Kieff, 1996). The EBV outer lipoprotein envelope contains a number of external protein spikes that are intimately associated with the interaction and infection of susceptible cells. One such glycoprotein to be described later in greater detail, glycoprotein 350 (gp350), is critical for infectivity and is the most abundant protein on the EBV envelope. The EBV genome is a linear, double stranded molecule of 172 kilo-base pairs (kbp) containing many internal and terminal repeat sequences (IR and TR, respectively) (Kieff, 1996) (see Figure 1 for the circular EBV genome as Figure 2 will be shown later with the linear EBV genome and the gp350 protein). Strain variations between EBV isolates are sometimes characterized by differences in the number of internal repeat (IR) sequences. The IR sequences contain within them, perfect and/or IR domains that often code for repeat sequences in viral proteins and are thought to have origins in the cellular host DNA.

In contrast to other herpesviruses, the EBV genome may have evolved and incorporated some segments of host cellular DNA. Sequences found in normal cellular DNA, GGGGCAGGA, are known to also exist repeatedly in the EBV nuclear antigen-1 (EBNA1) gene and antibodies specific to this protein will cross-react with a number of cellular proteins. In addition to this, EBNA1 interacts with cellular proteins and may be interpreted that some components of this viral protein may have arisen from its cellular host (Wen, et al., 1990). Examples of viral proteins which resemble host proteins include BZLF1 which is related to the *fos/jun* family of oncoproteins, the viral BHFR1 gene is similar to the Bcl-2 oncogene (described later) and viral BCRF1 is similar in composition and activity to the human interleukin-10 (IL-10) protein (all EBV genes are described in greater detail in Kieff, 1996). The activities of some of these viral homologs may partially explain some of their viral functions. The Bcl-2 viral homolog BHFR1 may be associated

with giant follicular cell lymphomas while BCRF-1 has been shown to suppress certain immune functions like its human counterpart, hIL-10.

EBV is known to exist in two main forms; EBV-1 and EBV-2. Their differences lie in the amino acid sequence homology of its latent genes LP-2, -3A, -3B and -3C. In addition, while EBV-1 is found most commonly in developed geographical areas, EBV-2 is found mostly in Asia and underdeveloped areas of Africa. To this end, EBV-1 monoclonal antibodies (mAbs) to EBNA1, -3A, -3B and -3C will often cross-react with other strains of EBV-1 but less often with those antigens of EBV-2. In contrast, mAbs generated from African/Asian isolates will generally react with both EBV-1 and EBV-2 viral proteins. Strain variation will also exist between viruses isolated from diffferent geographic locations. Recent evidence from patients with HD and HIV-1 infection appears to demonstrate geographic/strain variation as it pertains to mutation of the EBV-LMP-1 protein (Khanim et al., 1996 and Dolcetti et al., 1997). Dolcetti (1997) found that HIV-1 infected patients had, with greater frequency, similar mutations in LMP1, mutations that were not found with similar frequency outside this population. However, even with these differences, EBV-1 and -2 remain remarkably similar when their genomes are digested with a number of different restriction endonucleases.

Circular EBV Genome



1.3 EBV INFECTION.

EBV primarily infects epithelial cells and lymphocytes (B-lymphocytes, tonsillar lymphocytes and fetal-chord blood-lymphocytes). Being shed transiently in saliva, most EBV is found in peripheral blood lymphocytes, remains in a stable latent form and is generally non-replicating. These infected cells express six different nuclear proteins (EBV nuclear antigens, EBNAs), two different integral membrane proteins (LMPs), and two small-nonpolyadenylated RNAs (EBERs). The expression of these viral genes immediately after infection allows the B-cells to proliferate within 2-3 days. Infection of B-cells by EBV makes them respond to a number of mitogens and enhances their activity, mimicking the proliferation of lymphoblastoid cell lines (LCLs, Kieff, 1996).

Infection during the early years of life generally results in a self-limiting disease characterized as infectious mononucleosis. This is usually a transient infection of 1-2 months associated with virus replication in pharyngeal epithelial cells and polyclonal proliferation of B-lymphocytes. Fever, pharyngitis, lymphadenopathy and splenomegaly also characterize infectious mononucleosis. Following infection, serum IgG is responsive to viral capsid and nuclear antigens and remains present for many years. Resolution of active B-lymphocyte infection is usually attributed to cytotoxic T-lymphocytes and natural killer cells that are stimulated by anti-viral cytokines. With the interruption of active viral proliferation, the EBV genome sequesters itself in the nucleus of pharyngeal squamous epithelial cells or residual B-lymphocytes. Infection of squamous epithelial cells has been attributed to circulation of infected B-lymphocytes through the pharyngeal lymphoid tissues (Nemerow and Cooper, 1984 and Kieff, 1996).

Individuals infected with EBV generally carry lymphocytes capable of expansion *in vitro* or lymphocytes that undergo lysis. Lysis of infected cells releases progeny virions capable of infecting other cells. Contrary to the expansion of LCLs *in vitro*, growth of Burkitt's lymphoma (BL) cells may also occur through the genetic rearrangement of the *c-*

myc oncogene. In these BL cells, EBV may either integrate into the host genome or remain as an episome in the nucleus and often these cells only express the EBNA1 viral gene product. If the viral DNA remains as an episome, LCL cells may contain up to 10 copies in the nuclei while BL cells typically contain many more. In tissue culture, EBV-DNA may be present as either integrated or episomal (Kieff, 1996). Burkitt's lymphoma cells also differ in that they generally do not express the same cell surface activation markers, as do activated B-lymphocytes. In some cases however these BL cells may proliferate *in vitro* and become permissive to virus replication like other infected B-cells. Infected lymphocytes have characteristic alterations or cytopathic changes including formation of intra-nuclear inclusions, margination of nuclear chromatin, assembly of capsids within the nucleus, budding of virus through the nuclear membrane, inhibition of cellular protein synthesis and formation of cytoplasmic vesicles (Kieff, 1996).

1.3.1. LATENT INFECTION OF B-LYMPHOCYTES.

Early after infection of B-lymphocytes, cells usually undergo a transient state of activation characterized by the secretion of cytokines (e.g. interferon-α) and the production of immunoglobin molecules (Tanner et al., 1987, Delcayre et al., 1993 and Martin et al., 1994). Cellular stimulation of this sort can be accomplished by cross-linking CD21 molecules on the surface with a number of other associated plasma membrane receptors and may be interpreted as infected cells attempting to inhibit further infection of adjacent cells (Martin et al., 1994).

During latent infection of B-lymphocytes, up to 11 EBV related genes are activated including 6 EBNAs, 3 LMPs and 2 EBERs. In infected cells, the linear viral DNA circularizes in the nucleus through the use of the cellular transcription machinery since the

virus does not carry its own DNA polymerases (Alfieri et al., 1991). The use of cellular transcription materials is facilitated by the presence of upstream transcription factors including ATF, Sp1, NF-κB and TATA box elements in the 5'-untranslated region of many EBV genes (Sample et al., 1992, Howe et al., 1993 and Sugano et al., 1997).

With the expression of EBV latent proteins EBNA-1, EBNA-2, EBNA-3A, -3B, -3C, EBNA1 LMP-1, LMP-2A, -2B, BHRF1, BARFØ and EBERs, the infected B-lymphocyte now resembles an antigenically/mitogenically activated B-cell. One characteristic of EBV latency and transformation is the necessity of autocrine growth stimulation through cytokines such as TNF-β, IL-5, -6 and IL-10. These soluble proteins have been shown to facilitate LCL expansion and are present in B-lymphocyte cultures (Tosato et al., 1990 and Tanner et al., 1992).

1.3.2. Lytic Infection of B-Lymphocytes.

Treatment of EBV-infected cells with transforming growth factor-β, protein-kinase-C, calcium ionophore agents and Ig crosslinking agents will activate latent EBV. Following treatment, a number of EBV promoters become activated and transcribe and translate up to 30 different EBV genes. Gene activation occurs at many different sites along the EBV genome. Lytic genes occur randomly along the length of the genome, are intermingled with latent genes and may be differentially spliced to produce a number of different products (Kieff, 1996).

The earliest of these genes encode proteins capable of augmenting transcription and trans-activating other EBV-specific genes. Two very abundant proteins are BALF2 and BHRF1 (Kieff, 1996). While the BALF2 protein has DNA binding activity and is important in DNA replication, BHRF1 localizes in the nuclear and rough endoplasmic reticulum (rER) membranes. BHRF1 shares amino acid homology with the cellular bcl-2

protein, a protein that inhibits cellular apoptosis during viral replication and it is therefore thought that BHRF1 plays this same role during EBV production and assembly (Kieff, 1996).

Late lytic genes code mostly for viral structural protein. These include all core proteins and plasma membrane glycoproteins. The most important of these is the membrane associated glycoprotein-350 (gp350) encoded by the BLLF1 gene (described later). In addition to structural proteins, the late lytic phase also encodes BCRF1, a protein with ~80% homology to hIL-10. The human form is a major negative regulator of immune activity and BCRF1 may induce a similar immunosuppression during virus production from infected cells.

While the mechanisms used by EBV during virus packaging and shedding have not been well characterized, studies from other herpesviruses do provide some clues as to their role. Following initial viral activation and replication, the structural proteins and genome migrate to the Golgi apparatus and the rER where proteins are glycosylated and virus packaging occurs through formation of vesicules that migrate to the plasma membrane. There, the virions collect any number of cell surface proteins along with gp350 and are shed in a manner similar to HIV (Kieff, 1996).

1.4. EBV-GP350 AND ITS ROLE IN VIRAL INFECTION.

Infection by EBV is directed by its major envelope glycoprotein gp350. Interaction of gp350 is limited to cells expressing the 140-kDa cell surface receptor CD21/CR2, the receptor for the C3d component of complement. Expression of this receptor on heterologous cells is sufficient to allow viral interaction. After interaction with CD21, the virion induces the membrane aggregation of CD21 and the internalization of the CD21-virion complex through the fusion of the cellular and viral plasma membranes (Nemerow and Cooper, 1980, Carel et al., 1990 and Birkenbach et al., 1991). The internalization process is mediated by the gp350 hydrophobic-and amphipathic domains that facilitate the virus-cell membrane interaction (Nemerow and Cooper, 1980).

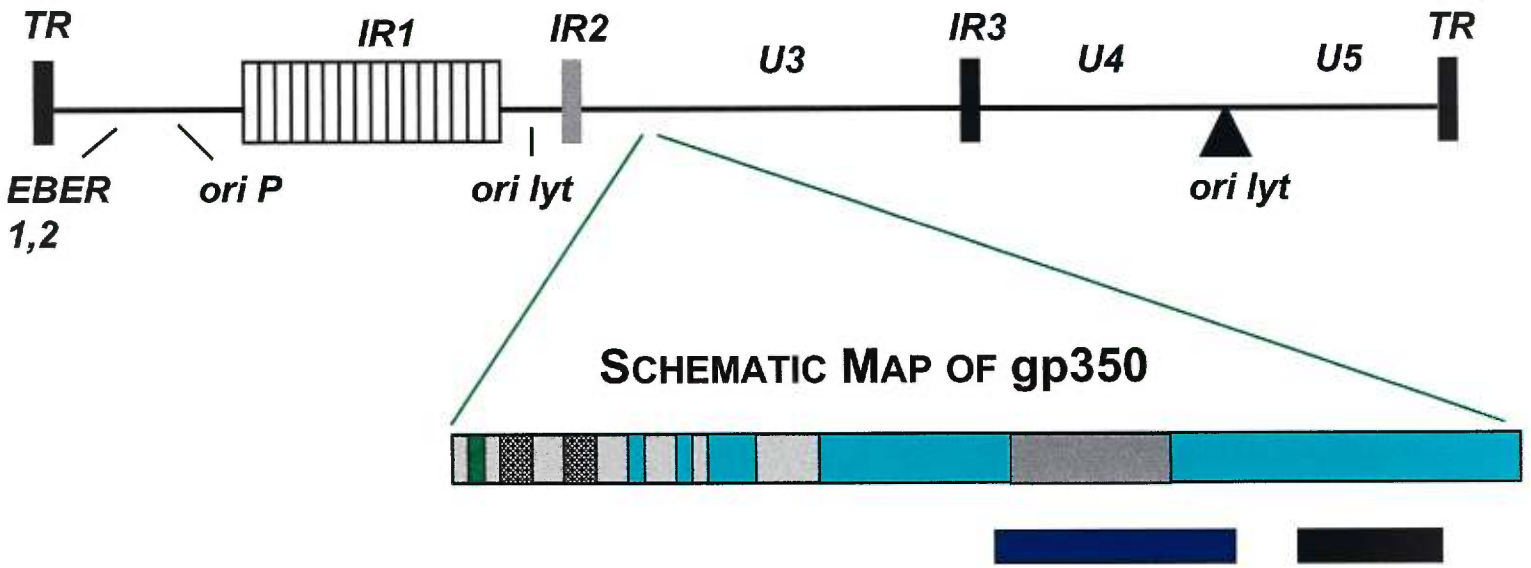
Membrane fusion being completed, the capsid negotiates its way to the nucleus by mechanisms which are not yet clearly defined but which probably involve cytoskeletal proteins similar to the movement of adenovirus to the nucleus (Dales and Chardonnet, 1973). Entry into the nucleus produces a number of circular EBV episomes that are free of viral proteins. The subsequent expression or inhibition of viral proteins may ultimately be determined by the cellular state of activation. Studies tend to suggest that the cellular activation state will determine whether EBV initiates a lytic or latent state (Miller et al., 1981).

1.5. CR2/CD21 AND ITS INTERACTION WITH EBV-gp 350.

The most abundant envelope glycoprotein on the surface of the EBV virion weighs 350-kDa and is commonly called gp350. This is the most important component involved in the infection of cells expressing CD21/CR2. Gp350 is made from the same open reading frame as gp220, another EBV surface glycoprotein that is of lower molecular weight due to the loss of an exon (Hummel et al., 1984 and see Figure 2 for the linear EBV genome and a schemativ of the gp350 protein). It is now recognized that both gp350/220 and the C3d component of complement bind to the same area of CD21/CR2 designated by the amino acid sequence EDPGKQLYNVEA. Removal or exchange of even two amino acids from the gp350/220 molecule interacting with VE are sufficient to inhibit interaction with cellular CD21/CR2 (Carel et al., 1990 and Birkenbach et al., 1991). The amino acid sequence of CD21/CR2 produces a protein with a signal peptide like area, a long hydrophobic domain with N-linked glycosylation sites and a carboxy terminal transmembrane anchor sequence (Beisel et al., 1985).

The interaction of CD21/CR2 and EBV-gp350 has been well characterized and involves specific amino acid residues. Two short consensus repeat modules (SCRs) are known to be critical for binding; mutation of these 60-70 amino acid sequence SCRs interrupts the interaction of CD21/CR2 with C3d or gp350 (Molina et al., 1995). These SCRs are not unique to CD21/CR2, but are found as modular units in the receptors for IL-2, the β-subunit of clotting factor XIII and a number of other complement receptors (Hourcade et al., 1989). Each SCR contains a β-sandwich with two β-strands on one face, three β-strands on the other face and a compact globular hydrophobic core (Martin et al., 1994). Interaction of C3d or EBV with CD21/CR2 occurs specifically through SCR-1 and SCR-2. Fusion of SCR-1/SCR-2 with IgG1 interacting with CD21/CR2 can block EBV attachment to its allied membrane receptor (Moore et al., 1991).

LINEAR EBV GENOME:



(Lees et al., 1993)

Initially discovered on B-lymphocytes (Nemerow et al., 1984 and Fingeroth et al., 1985), CD21/CR2 is now known to be present on a number of other cells. T-lymphocytes have been shown to express the 140 kDa membrane receptor and interaction with EBV +or gp350 can lead to cellular activation and aggregation (Fingeroth et al., 1988, Sauvageau et al., 1990, Hedrick et al., 1994, Luxembourg and Cooper, 1994 and Prodinger et al., 1996). Kaufman-Patterson (1995a, 1995b) found that infection of T-lymphocytes with EBV caused cells to proliferate in response to CR2 ligation without the need for exogenous cytokines and to modulate the expression of critical T-cell receptor molecules.

Expression of CD21/CR2 has also been demonstrated on epithelial cells (Birkenbach et al., 1991), endothelial cells (Jones et al., 1995), follicular dendritic cells (Liu et al., 1997), astrocytes (Gasque et al., 1996) and monocytic cells (Inada et al., 1983, Revoltella et al., 1989 and Gosselin et al., 1991). These reports demonstrate not only that the receptor exists on these cell lineages but that interaction with EBV or gp350 alone is sufficient to modulate normal cellular activity. While endothelial cells have been shown to support viral infection through the expression of EBERs (Jones et al., 1995), in some cases interaction with CD21/CR2 will modulate the transcription and translation of cytokines without the need for overt viral infection. (Gosselin et al., 1991, 1992a and 1992b).

CD21 may also play a role in antigen presentation. In addition to the recent evidence of a CD21/CR2 receptor on dendritic cells, questions arise as to EBV's role in germinal center development as dendritic cells play a major role in antigenic presentation and stimulation (Liu et al., 1997). Evidence using Influenza virus/complement complexes demonstrated that class II restricted T cell clones would have augmented responses to antigen presentation compared to those cells without CD21/CR2 (Boackle et al., 1997). In addition, the authors found that increased CD21/CR2 expression produced enhanced

antigen presentation responses compared to cells expressing fewer receptors (Boackle et al., 1997).

Signaling through CD21/CR2 following interaction with EBV-gp350 or C3d involves specific signal transduction pathways (Fearon and Carter, 1995). Previous data indicates that ligation of CD21/CR2 with activated protein kinase C and the *lck* tyrosine kinase leads to the phosphorylation of a short CR2 cytoplasmic component and the possible mobilization of Ca^{2+} ions (Barel et al., 1986, Chagelian and Fearon, 1986, Dugas et al., 1988, Cheung and Dosch, 1991, Aquino et al., 1993 and Tanner et al., 1996). The receptor-ligand interaction may also involve cellular membrane components such as CD19, Leu-13, and TAPA-1 (target for antiproliferative antibody-1). With CD21/CR2 having a short intracytoplasmic carboxy terminal tail, the signal transduction mechanism probably does not involve this receptor directly but may occur through its association with the other above-mentioned receptors. Fearon and Carter (1995) found that one or all of these receptors may associate extracellularly with CD21/CR2 and were more likely the source of any intracellular activation signal.

Bradbury (1992) and Matsumoto (1991 and 1993) also found that direct activation of CD19 via cross-linking through EBV was sufficient to activate phospholipase-C and phosphotidyl-inositol-3-phosphate in B-lymphocytes. These studies demonstrated that interaction of CD21/CR2 with CD19 was occurring within the plasma membrane and involved other proteins which acted as peptide-bridges between the two receptors. The authors also demonstrated that CD19-TAPA-1 and CD21/CR2-CD35 interacted extracellularly, while CD19 and CD21 interacted in either the extracellular or transmembrane region. An elegant co-receptor model is provided by Doody (1996); the authors provide an opinion regarding the cellular activation of signaling molecules through extracellular or transmembrane interaction of CD21/CR2 with TAPA1, Leu-13 and CD19. In all these possible forms of extracellular co-ligation, cellular activation would always be

the end result.

Using specific peptides, activation through CD21/CR2 is now known to result in the phosphorylation of pp105, and in CD21/CR2 interaction with p53, p68 (a Ca^{2+} binding protein) and ribonucleoprotein p120 (Frade et al., 1992, Barel et al., 1995 and Bouille et al., 1995). In these reports, cellular activation was found to be transmitted directly through a 34 amino acid sequence within the intracellular component of CD21/CR2 arguing against the assistance of CD19, TAPA-1 or Leu-13 (Balbo et al., 1995). In addition, Bouille (1995) found that phosphorylation of pp105 could occur through both a CD19-dependent and independent pathway. More recent work from this group now demonstrates CD19 independent activation of phosphatidylinositol-3-kinase (PI-3-K) through CD21/CR2 in B-lymphocytes (Bouille et al., 1999). Earlier studies using B-lymphocyte surface molecules also found that interaction with IgM produced increased intracellular free Ca^{++}, tyrosine phosphorylation and association of PI-3-K with CD19 (Matsumoto et al., 1993). With these conflicting studies on the role of CD21/CR2, the signal transduction pathway(s) need to be elucidated. It is known that multiple intracellular activation sequences proceed the interaction of CD21/CR2 with several extracellular proteins.

1.6. CYTOKINES.

Cytokines comprise a network of soluble mediators released predominantly by hematopoietic cells; in general, cytokines enhance or suppress cell functions that act to eliminate pathogenic agents (ie: bacteria, viruses) tumors or toxins. Production of different interleukins (IL's), interferons (IFN's), colony stimulating factors (CSF's), transforming growth factors (TGF's) or tumor necrosis factors (TNF's) have been shown to increase rapidly and to very high levels in response to pathogenic agents. These molecules have been shown to be secreted by a number of cells including T- and B-lymphocytes, monocyte/macrophages, natural killer cells, neutrophils, fibroblasts, dermal dendritic, leukemic and neural cells (astrocytes and microglia, Arai et al., 1990). Cytokines are polypeptide hormones that upon secretion can act locally or at distant sites and interact with specific receptors to alter cellular behaviour. The precise biological effect of any cytokine will be determined by the millieu in which it acts and the other cytokines present. Cytokine release causes a number of events including inflammation, fever and other pyrogenic phenomena, induction of an antiviral state, cellular differentiation and stimulation of cell proliferation.

1.6.1. TUMOR NECROSIS FACTOR-α (TNF-α).

One of the major cytokines produced by immune cells is one that has also been found to be deleterious at high levels, tumor necrosis factor-α (TNF-α). Found over two decades ago to mediate nonspecific tumor cell killing, TNF-α was purified from endotoxin stimulated mice and determined to be the cause of wasting and anorexia in these animals (Beutler and Cerami, 1989 and Rink and Kirchner, 1996).

TNF-α is a non-glycosylated ~17-kDa protein produced mainly by activated monocyte/macrophages and in smaller quantities by lymphocytes, endothelial cells, astrocytes and natural killer cells. In serum, TNF-α is not isolated as a monomer, but as a tightly packed immunoreactive trimer of ~50 kDa. TNF-α is capable of stimulating its own production, as well as IL-1, IL-6, IFN-β, CSFs and other growth factors, inflammatory mediators, cell adhesion molecules, major histocompatability proteins, cellular receptors, viruses and acute phase proteins. Significant intracellular accumulation of TNF RNA has been observed in stimulated cells demonstrating translational control. Lonneman (1989) found that IL-1α, IL-1β and TNF-α activation by endotoxin produced differential kinetics of both RNA and protein. Espel (1996) found that MHC class II interaction on monocytes might be another level of control. The authors found that interaction of MHC class II receptors with toxic shock syndrome toxin-1 (TSST-1) of *staphlococcus aureus* resulted in enhanced secretion of TNF-α; they found a four-fold increase in translational efficiency compared to non-TSST-1 treated cells.

More generally, TNF-α stimulates glycerol release from adipose tissues, suppresses adipocyte differentiation and activates neutrophil cytotoxicity, degranulation and adherence. TNF-α is a potent endogenous pyrogen; it stimulates bone resorption, degradation of cartilage and is directly cytolytic to many cells. Both the 55 and 75 kDa subunits of the TNF-α receptor are found on virtually all cells except erythrocytes and both

receptors bind TNF-α with high affinity. The stimulation of either subunit with specific antibodies is sufficient to induce a wide range of TNF-α dependent phenomena (Vilcek and Lee, 1991). The cytolytic activities of TNF-α are due not only to the activation of particular signal transduction pathways, but also due to the capacity of TNF-α to physically enter the cell membrane, disrupt normal membrane permeability and destroy ionic differences (Kagan et al., 1992).

Although the signal transduction pathway utilized by this cytokine-receptor system is not well understood, the interaction of TNF-α with its receptors mediates a number of activities. The receptors themselves have no intrinsic protein kinase C (PKC) activity but a number of PKs become activated within minutes of TNF-α/receptor interaction. In PKC-depleted cells, LPS is capable of inducing TNF-α production. However specific analysis found only selective depletion of certain PK isoforms, but not the ξ isoform could not suppress LPS induced TNF-α production (Fujihara et al, 1994). TNF-α addition to monocytes also induces cAMP production, albeit less quickly than PK (Shirakawa and Mizel, 1989).

TNF's most important inducer, LPS, through its interaction with CD14 induces CD14s interaction with other LPS binding proteins (septins) such that activation of TNF results (Ziegler-Heitbrock et al., 1993). Other data indicates that activation of TNF-α may involve other signal transduction pathways including phosphatidylcholine-specific-phospholipase-C (PC-PLC) producing an active cellular ceramide (Schutze et al., 1992). Recently, activation of TNF-α was found to be differentially inducible in both T and B-lymphocytes and dependent on both NFATp (nuclear factor of activated T-cells) and ATF-2/JUN (active T cell factor) via a cAMP pathway (Tsai et al., 1996). From these extensive studies it is apparent that the true mechanism underlying activation of TNF-α is cell and mitogen specific.

1.6.2. INTERLEUKIN-1 (IL-1).

Formerly called endogenous pyrogen, IL-1 is a potent inducer of fever, sleep, inflammation and release of acute phase response proteins of hepatocytes. IL-1 induces its own production, along with the production of IL-6, IL-2, IL-4, GM-CSF and TNF-α (Dinarello, 1996). Although IL-1 is able to mediate a number of neurological functions including sleep induction, decrease in appetite, release of adreno-corticotropin hormone and brain prostaglandins, one of the most interesting aspects of its biology is its ability to traverse the blood brain barrier. Therefore, its effects on the brain are thought to be due to its ability to activate the release of specific proteins from brain endothelial cells, a cell type that expresses receptors for both IL-1 subtypes (Pober and Cotran, 1990).

Transcriptional activation of IL-1 has not been completely characterized. Analysis of agents capable of stimulating IL-1 production indicates that it is very responsive to bacterial endotoxin; treatment of cells with the protein synthesis inhibitor cycloheximide (CHX) causes IL-1 superinduction. Furthermore, addition of actinomycin-D (Act-D) to cell cultures stimulated with LPS found that IL-1β RNA half life was shortened and may involve selective degradation (Fenton et al., 1988). Rapid degradation of cytokine RNA transcripts has also been observed for TNF-α, GM-CSF and IFN-β (Arai et al., 1990).

The effects of IL-1 are mediated by specific receptors which are responsive to both IL-1 subtypes (-α and -β); these receptors are found on many cell types including monocyte/macrophages, T and B-lymphocytes, fibroblasts, hepatocytes and endothelial cells. IL-1 induces a number of intracellular effects that are usually produced following the production of arachidonic acid metabolites including prostaglandins and other lipoxygenase byproducts (Dinarello, 1996). The two IL-1 receptor types are 68 and 80-kDa; they both contain extracellular domains present in the Ig superfamily and although they can interact with both IL-1α and--1β, demonstrate only 30% extracellular homology. The intracellular domain does not have protein kinase activity per se but can be

phosphorylated at serine and threonine residues. It is not known if phosphorylation of these sites is required for biological activity although IL-1 and its receptors can activate the protein-kinase-A signal transduction pathway. Evidence also indicates that once IL-1 binds to its receptor, the receptor-ligand complex can be directly translocated to the nucleus. Furthermore, this entry occurs prior to any IL-1 induced gene activation events (Curtis et al., 1990).

As our work concentrated on the IL-1β subtype, the remainder of this section will concentrate on this, more information on IL-1α can be obtained from Dinarello, 1996. Many groups have analyzed the differential activation of IL-1β and found that LPS-activated monocytes produce significant amounts of protein (Turner et al., 1989). One of the most interesting aspects of IL-1β gene activation is that both transcription and translation are under independent control (Schindler et al., 1990); significant amounts of IL-1β RNA can accumulate intracellularly without any appreciable secretion or membrane bound protein forms. With the cloning of the IL-1β, transcriptional activation mechanisms are now being more closely analyzed (Bensi et al., 1990 and Hiscott et al., 1993).

It is now known that transcriptional activation of the IL-1β promoter requires the presence of NF-κB, CREB, NF-IL6 and AP-1 transcription factors. Cross-linking of ICAM-1 (CD54) will induce AP-1 mediated activation of IL-1β (Koyama et al., 1996) while LPS and PMA appear to mediate their effects through NF-IL-6, NF–κB, and CREB (Gray et al., 1993, Hiscott et al., 1993, Shirikawa et al., 1993, Cogswell et al., 1994, Tsukada et al., 1994 and Pan et al., 1996). Keratinocytes express IL-1β and stimulation of these cells with TNF-α results in increased IL-1βCAT activity through a Sp-1 dependent transcription binding site (Husmann et al., 1996) demonstrating that transcriptional activation of this gene potentially requires multiple transcription factors which are differentially activated.

To examine this regulation more closely, Kaspar and Gehrke (1994) found that

C5a or LPS treated PBMC accumulate significant amounts of intracellular IL-1β mRNA without secretion or accumulation of IL-1β protein. In this study, IL-1β mRNA was not released from polyribosome complexes suggesting a second signal is required to liberate this mRNA from these complexes. Post transcriptional cytoplasmic IL-1β mRNA accumulation is not a new phenomenon since Schindler (1990) found that transcriptional activation of PBMC by LPS or *stapholococcus epidermidis* resulted in intracellular accumulation of transcripts which were not translated into protein.

Post-transcriptional regulation may also occur at the level of mRNA stability. The 3'-untranslated region (UTR) of IL-1β, when genetically introduced into the 3' UTR of CAT RNA produced significantly less transcription with decreased RNA stability. When stimulated with LPS this hybrid CAT RNA 3'-IL-1β UTR was found to have a significantly longer half-life compared to constructs lacking the 3' IL-1β UTR (Kern et al., 1997). Multiple levels of IL-1β regulation are not surprising when one considers the potentially harmful effects of IL-1β over-expression. In this context, IL-1β gene expression can be superinduced in monocytes by CHX addition indicating that RNA is transiently produced and post-transcriptionaly degraded or repressed by a newly synthesized protein (Fenton et al., 1987 and 1988). Furthermore, a cell-free plasma form of IL-1β called the IL-1β receptor antagonist protein exists. This protein is 26% homologous to the immunoreactive IL-1β protein and is capable of interacting with both IL-1β receptor types without inducing cellular activation signals (Carter et al., 1990). This antagonist protein in the plasma may serve as yet another level of IL-1β regulation.

Produced by a number of cells including monocyte/macrophages, T- and B-lymphocytes, fibroblasts, neutrophils and nervous system microglia and astrocytes, IL-1 affects almost all biological tissues and processes (Dinarello, 1991 and Mizel, 1989). The premature IL-1β precursor protein is generated as a 33-kDa precursor that is subsequently

cleaved by the IL-1β-converting enzyme (ICE). While the steady state levels of interleukins *in vivo* is low, production of IL-1, in response to inflammatory agents, bacterial, or viral infection, causes a rapid increase in serum levels and a cascade of effects whose activities are evident in many tissues.

1.7. Nuclear Factor Kappa B (NF-κB).

NF-κB was originally described as a factor interacting with the immunoglobin-κ light chain gene enhancer (Baeuerle and Baltimore, 1996). Although originally identified in B-lymphocytes, this family of proteins is now known to exist in a number of different cell types (Verma et al., 1995, Baeuerle and Baltimore, 1996 and Baldwin, 1996). NF-κB is a family of structurally and functionally related peptides that regulate transcription of viral genes (HIV, CMV, SV40 and Adenoviruses), genes coding for cell surface receptors (MHC class I, IL-2 receptor-α chain, T cell receptor-β and -β_2 microglobulin), VCAM, cytokine genes (IFN-β, GM-CSF, G-CSF, IL-1β, -2, -6 and TNF-α, -β) and genes coding for several transcription factors (IRF-1, IκB-α, *c-myc* and NF-κB) (Baldwin, 1996).

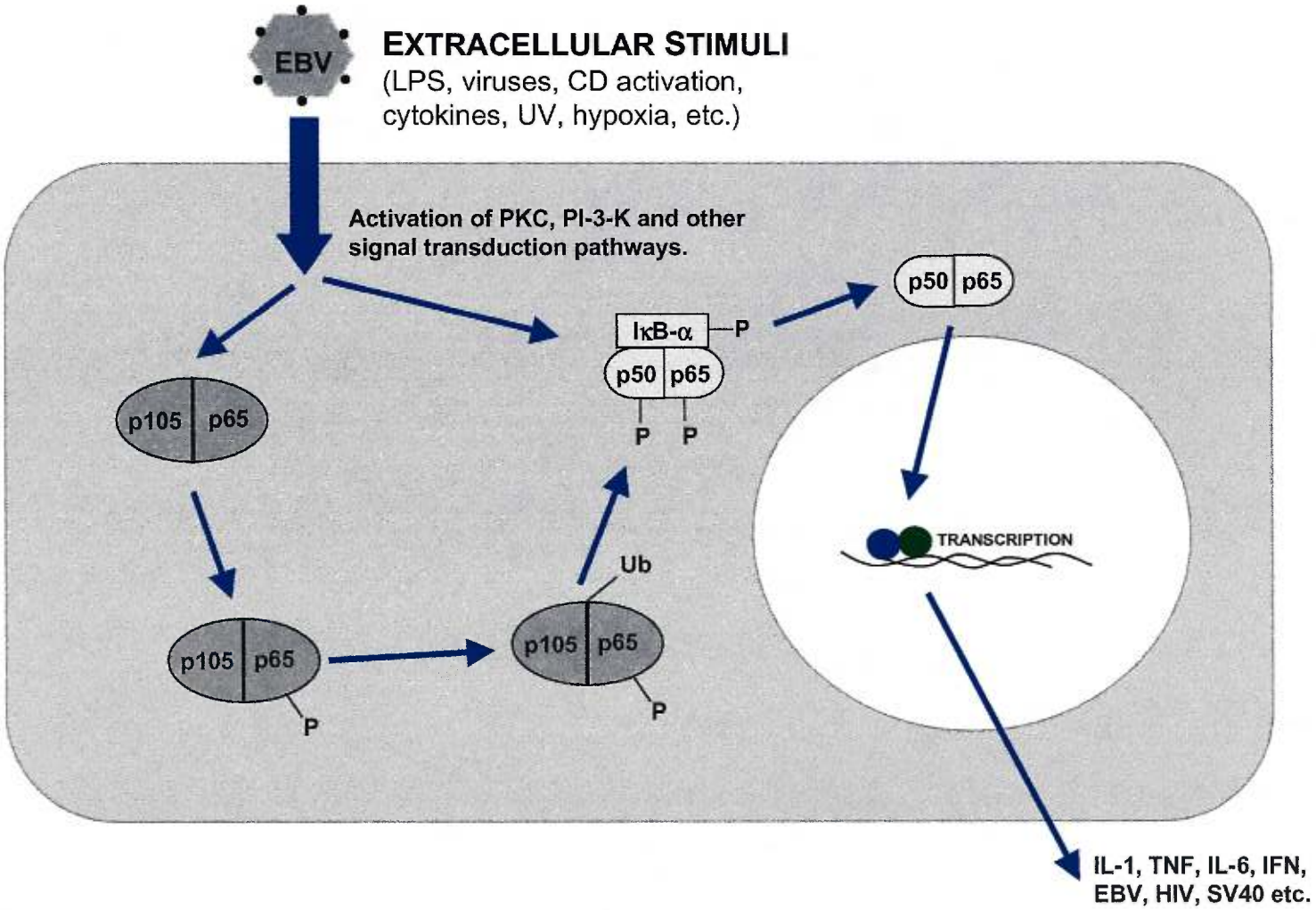
Originally described as two proteins (50- and 65-kDa), cloning revealed that a number of proteins shared significant homology with these peptides in the amino terminal DNA binding region. This protein family shares homology with the *rel* family of oncoproteins found in the avian reticuloendotheliosis virus (Rev-T), and the Drosophila developmental morphogen *dorsal* (Verma et al., 1995 and see Figure 3). On the basis of this homology, the NF-κB family now consists of a number of peptides ranging in molecular weight from ~49 kDa to 100 kDa.

NF-κB is found not only in B-lymphocytes, but pre-exist in the cytoplasm of cells types coupled to the inhibitor IκB (inhibitor of κB). Activation of T and B-lymphocytes, monocyte/macrophages and fibroblasts by a number of agents including phorbol esters, ionizing radiation, oxygen radicals, LPS, viruses (HIV-1, CMV, HHV-6, HBV, HSV-1 and HTLV-1) or cytokines (IL-1 and TNF-α, -β) liberates the -κB binding proteins from IκB, causing them to translocate to the nucleus where they interact with DNA (see Figure 3). It has been established that the 65-kDa subunit is the subunit interacting with cytoplasmic IκB. The activation of the DNA binding subunits and their translocation into the nucleus is mediated by the dissociation of the cytoplasmic complex. Once translocated to the nucleus, the p50 and p65 peptides interact in different combinations to a decameric DNA sequence containing the consensus motif 5'-GGGRNN(YYC)C-3' (where Y denotes pyrimidines, R denotes purines and N is any nucleotide).

Data suggests that variations in DNA affinity, heterodimer formation and induction kinetics may contribute to both positive and negative transcriptional control. In addition, various -κB regulated genes containing slightly different -κB DNA sequences may be differentially regulated by different NF-κB subunit interactions. Other -κB like clones have recently been isolated, demonstrate significant homology to this protein family and have been cloned from various cells. Thus, genes containing -κB sequences in their promoter, apart from possibly being transcriptionally regulated by other additional factors, can be differentially regulated by a number of different -κB subunit combinations.

Although these proteins were originally thought to activate immunoregulatory genes, NF-κB is now known to interact with widely different promoters including its own subunit promoters. Cytokine genes such as TNF-α, IFN-β, GM-CSF, G-CSF, IL-1β, IL-6, IL-2 and TNF-β have all been shown to contain DNA sequences capable of binding NF-κB proteins (Baeuerle and Baltimore, 1996, and Baldwin, 1996).

NUCLEAR FACTOR-KAPPA B REGULATION



This family of transcription factors is involved in cell survival. Transgenic and knockout mice lacking particular subunits of NF-κB have embryonic pathologies including B and T-cell deficiencies, diminished thymocyte development, altered lymph node architecture, skin defects, multiple organ inflammation, modified granulopoiesis and severe hepatocyte apoptosis (Baeuerle and Baltimore, 1996). From this it is apparent that this family of transcription factors initially described as a mediator of immune activation plays a much larger role in development and cell death.

1.8. RESEARCH OBJECTIVES.

The objective of the work presented in this thesis was to analyze the mechanisms underlying the immune-modulation during interaction of hematopoietic cells with Epstein-Barr virus and its major envelope glycoprotein gp350. Our goal was to examine how the transcription and translation of interleukin-1β, interleukin-6 and tumor necrosis factor-α genes was modulated upon interaction with EBV in culture. This analysis progressed to examine the transcriptional activation of the IL-1β, IL-6 and TNF-α genes as it pertained to the stimulation via the NF-κB family of transcription factors.

2.0 MANUSCRIPTS

Epstein-Barr virus envelope glycoprotein gp350 induces NF-κB activation and IL-1β synthesis in human monocytes-macrophages involving PKC and PI3-K

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ABSTRACT **Epstein-Barr virus (EBV) is a highly immunotropic human herpesvirus with oncogenic potential and is involved in numerous pathologies. EBV utilizes its major envelope glycoprotein gp350 to bind to its receptor CR2/CD21 on target cells for initiating the infection. We have previously shown that EBV is able to modulate transcription and translation of a number of cytokine genes via its gp350-mediated binding to this receptor. However, the effects of the binding of purified gp350 to CR2/CD21 on plastic-adherent monocyte-macrophages (AMM) have not been investigated. These cells are a rich source of potent proinflammatory and immune-modulating cytokines, and express low levels of CR2/CD21. We show here for the first time that recombinant gp350 (rgp350) causes production of the potent proinflammatory cytokine IL-1β in human AMM. Surprisingly, rgp350 is comparable in this capacity to the phorbol ester 12–0-tetradecanoylphorbol 13-acetate. This induction of IL-1β production was accompanied by increased steady-state levels of its mRNA in gp350-treated AMM, and was dependent on the specific binding of rgp350 to the EBV receptor CR2/CD21. We also show that the signaling pathways resulting in the induction of IL-1β synthesis by rgp350 required protein kinase C and phosphatidylinositol 3,4,5 triphosphate kinase activities and occurred via activation of the NF-κB family of transcription factors.—D'Addario, M., Ahmad, A., Xu, J. W., Menezes, J. Epstein-Barr virus envelope glycoprotein gp350 induces NF-κB activation and IL-1β synthesis in human monocytes-macrophages involving PKC and PI3-K. *FASEB J.* 13, 2203–2213 (1999)**

Key Words: EBV gp350 · interleukin 1β · signal transduction · transcription factors

EPSTEIN-BARR VIRUS (EBV) is a ubiquitous lymphotropic human herpesvirus associated with numerous pathologies including infectious mononucleosis, African Burkitt's lymphoma, nasopharyngeal carcinoma, oral hairy leukoplakia, B cell lymphomas in the immunocompromised host, and Hodgkin's and non-Hodgkin's lymphoma (reviewed in ref 1). Primary infections with EBV occur via epithelial cells, which subsequently release virus and lead to infection of B lymphocytes. EBV binds to and infects cells through complement receptor type II (CR2 or CD21), which is the natural ligand for the C3d fragment of the third component of complement (2–5). Glycoprotein (gp)350 is the major envelope glycoprotein of EBV; it specifically binds to CR2/CD21 and initiates the viral infection process (5, 6). Apart from initiating this process, gp350 is the major target protein for EBV-specific humoral and cellular immune responses; many anti-EBV subunit vaccines currently under human trials are based on this glycoprotein (reviewed in refs 7, 8).

CD21 belongs to a family of proteins containing short consensus repeats (SCRs), a structural module found in many other proteins associated with inflammation, tissue repair, and immune responses (reviewed in refs 2, 9). Although originally described on B-lymphocytes, where it represents an important component of the B cell antigen receptor signaling complex, CD21/CR2 has also been found on follicular dendritic and endothelial cells, thymocytes, T cells, epithelial cells, and monocytes (4, 10–15). Thus, EBV may infect and/or interact with cells of these diverse lineages and modulate their physiological activities.

Earlier studies from this laboratory have shown that EBV modulates the synthesis of proinflammatory cytokines interleukin 6 (IL-6), IL-1β, and tumor necrosis factor α (TNF-α) from human peripheral blood mononuclear (PBMC) and monocytic tumor cells (12, 16, 17). These effects of EBV on cytokine synthesis in human cells were dependent on the binding of the viral particles to the EBV receptors on the target cells, suggesting the involvement of gp350 in this process. More recently, Tanner et al. (18)

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reported the induction of IL-6 synthesis via protein kinase C (PKC) in human B cells. These studies indicate that gp350 can interact with CR2/CD21-bearing human cells and modulate the synthesis of cytokines in them. The effects of the potential interaction of gp350 with human monocyte-macrophages have not been investigated, however. These cell types play a pivotal role in the induction of immune and inflammatory responses, represent an important source of proinflammatory cytokines in the body, and express low levels of CR2/CD21 on their surface (12, 14; reviewed in ref 19). In this report we have addressed this issue and investigated the effects of purified recombinant gp350 (rgp350) on the synthesis of IL-1β in human plastic-adherent monocyte-macrophages (AMM). IL-1β is a potent multifunctional proinflammatory cytokine whose activities affect almost all other cell types (reviewed in ref 20). Apart from playing a crucial role in the regulation of immune and inflammatory responses, IL-1β can traverse the blood–brain barrier and therefore can also affect neurological functions (20). Our results indicate that synthesis of this important cytokine is up-regulated by rgp350 through pathways that involve enzymatic activities of PKC and phosphatidylinositol 3,4,5 triphosphate kinase (PI3-K) and activation of the NF-κB family of transcription factors. To our knowledge, this is the first report describing the immunobiological consequences of the direct interactions between purified rgp350 and human plastic-adherent blood monocyte-macrophages.

MATERIALS AND METHODS

Cell culture

PBMC were obtained from healthy donors by centrifugation of heparinized whole blood over a Ficoll-Paque density gradient (Pharmacia, Piscataway, N.J.). PBMC were plated on tissue culture dishes pretreated overnight with heat-inactivated fetal bovine serum (FBS) and allowed to adhere during an overnight incubation at 37°C. The culture medium used was RPMI 1640 (GIBCO BRL/Life Technologies, Burlington, Ont.) containing 10% (v/v) FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (pH 7.2), and 1 μg/ml gentamicin. Plastic-adherent monocyte-macrophages (AMM) were collected by gentle scraping with a rubber policeman. Cells were washed twice in RPMI (without FBS), resuspended in the culture medium at a concentration of 2×10^5 cells/ml, and used for present studies.

Cell treatments and preparation of samples

To see the effect of rgp350 on the synthesis of IL-1β in AMM, the glycoprotein was added to the (1 ml) cultures of AMM (2×10^5 cells/ml) at a final concentration of 100 ng/ml. This concentration was found optimal in pilot experiments to induce increased synthesis of this cytokine in PBMC (data not shown). The cultures were incubated at 37°C, in a 5% CO_2-containing humidified atmosphere for 36 h. Results from pilot experiments indicated that this was the time point at which the IL-1β synthesis reached maximum levels in PBMC when induced with rgp350 or UV-irradiated EBV (UV-EBV). After 36 h the culture supernatants were collected, centrifuged at $1200 \times g$ for 15 min at 4°C to remove cells and cellular debris, and stored at −80°C until used to determine IL-1β. The cells were washed with ice-cold phosphate buffered saline (PBS, pH 7.2) and processed for preparation of cytosolic fractions as described (21). Briefly, the cell pellet was swollen in five packed cell volumes of a hypotonic buffer containing 10 mM Tris (pH 8.2), 27 mM DMSO, 5 mM PMSF, 10 mM HEPES (pH 8.2), 0.75 mM spermidine, 1 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, and protease inhibitors as described earlier (21). The cells were lysed in a Dounce homogenizer (20–25 strokes) and the lysates were centrifuged at 45,000 rpm for 30 min at 4°C in a Ti-45 rotor (Beckman Ultracentrifuge, Fullerton, Calif.). The supernatants were collected, their protein concentration was determined by using the bicinchoninic acid (BCA) reagent kit (Pierce, Rockford, Ill.), and aliquots were stored at −80°C until examined. IL-1β concentrations were determined in 5 μg proteins of these cytosolic preparations.

As a positive control for the induction of synthesis of IL-1β, we treated AMM with TPA (12,0-tetradecanoyl phorbol 13-acetate, 25 ng/ml, Sigma, St. Louis, Mo.). In some experiments rgp350 was pretreated with monoclonal antibodies (mAbs) before adding to the cell cultures; this was done by incubating 100 ng of rgp350 with 5 μg of the antibody at room temperature for 15 min.

To see the effect of CR2-specific mAb OKB7 on the induction of IL-1β synthesis, this mAb was added to the cell cultures at a final concentration of 10 μg/ml. The cell cultures were kept at room temperature for 30 min before being treated with rgp350.

The cells were also treated with EBV (infectious or noninfectious; see below). For this purpose, the cell pellets were incubated with 100 μl of the virus preparation, vortexed gently, and incubated at 37°C for 1 h. After this, the cells were washed twice with the culture medium, resuspended at 2×10^5 cells/ml concentration, and incubated at 37°C for 36 h.

Unless stated otherwise, all treatments involved 2×10^5 cells in 1 ml volume of the culture medium and incubation for 36 h to determine IL-1β in the culture supernatants and cytosolic preparations or 6 h for quantitation of IL-1β mRNA in the cells.

Reagents and antibodies

To determine which signaling pathways were involved in the induction of IL-1β by rgp350, the cells were stimulated with this glycoprotein in the presence of specific inhibitors of various signal transduction pathway enzymes. The inhibitors used were tyrphostin AG1478 (an inhibitor of protein tyrosine kinases, epidermal growth factor receptor, and platelet-derived growth factor receptor, 100 μM), bisindoylmaleimide (a specific inhibitor of PKC, 5 μM), LY294002 (a specific inhibitor of PI3-K, 40 μM), staurosporine (a broad spectrum inhibitor of protein kinases, 50 nm), and MDL-12,330A-HCl (an irreversible inhibitor of adenyl cyclase, 1 mM). These inhibitors were all purchased from Calbiochem/InterScience (Markham, Ontario). The concentrations of the inhibitors used are shown in parentheses and are those recommended by the manufacturer.

Anti-CR2 mAb OKB7 was obtained from Ortho Diagnostic Systems (Raritan, N.J.), which neutralized the binding of rgp350 to target cells (Raji) at 5 μg/ml. Anti-gp350 mAb 2L10, which does not block rgp350 binding to CR2, was a gift from Dr. G. Pearson (Georgetown University, Washington,

D.C.); anti-gp350 mAb 72A1, which blocks binding of gp350 to CR2, was kindly provided by Dr. J. Gosselin (Laval University, Quebec, Canada).

Specific monoclonal antibodies for p50, p65, and c-Fos were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.); each was used in electrophoretic mobility shift assays (EMSA) at 1 μg per reaction.

The cell membrane-permeable synthetic peptide SN50 and its control SN50M were obtained from Calbiochem (San Diego, Calif.). This inhibitor peptide has been shown to block the nuclear translocation of p50 p65 and c-Rel in human monocytic cells, whereas the control peptide has no such effect (22). Incubation of both SN50 and its control with AMM cells for up to 5 days did not result in any significant toxicity.

EBV and rgp350 preparation

EBV was prepared as described previously (23). Briefly, the transforming strain (B95–8) of EBV (24) was obtained from cell-free supernatants of B95–8 cell cultures. Supernatants from 1-wk-old cultures of these cells were filtered through 0.45 μM filters (Nalge Labware, Corning, New York) and centrifuged at 45,000 × *g* for 90 min at 4°C. The viral pellets were resuspended in PBS to yield 500× concentration of virus as compared to the culture supernatants. The virus preparation was titrated by the induction of nuclear antigen (EBNA) in BJAB cells as described (23). The viral preparation used for these studies contained 2×10^5 EBNA-inducing units/ml. UV-inactivated virus was obtained by irradiating EBV for 60 min at 265 nm. After UV inactivation, it contained less than 10 EBNA-inducing units/ml.

The rgp350 preparation used was a gift from Dr. Andrew Morgan (University of Bristol, U.K.). It was produced in a mouse fibroblast cell line line (C127) after transfection with a bovine papilloma virus-based expression system (25). The gp350 produced, which lacked the membrane anchor region, was purified from the culture medium and further clarified using Sephacryl 5300HR and gelatin agarose (25). When examined on a silver stain gel, the protein gave bands of the expected molecular weight (data not shown) and its immunological activity was similar to the native EBV-gp350 (25). The endotoxin content of the EBV and gp350 preparations was determined and found to be less than 25 pg/ml according to a Limulus amebocyte lysate-based endotoxin detection kit (ICN Immunochemicals, Montreal, Canada).

Assay for cytokine concentration

Concentrations of IL-1β in the cell-free culture supernatants (secreted from) and in the cytosolic preparation (cytosolic or cell-associated form) were determined using a commercial ELISA Kit (R&D Systems, Minneapolis, Minn.) according to the manufacturer's instructions. The lowest limit of detection of IL-1β by this kit was 10 pg/ml and measured the 'free' forms of the cytokine.

RNA isolation

RNA isolation was performed using a modified guanidium thiocyanate procedure (26). Briefly, cells were collected by centrifugation (1200 × *g* for 10 min), rinsed in PBS (75 mM NaCl, 2 mM KCl, 8 mM NaH_2PO_4), and resuspended in 1 ml of solution D (4 M guanidium thiocyanate, 25 mM Na citrate, pH 7.0, 0.5% sarcosyl, and 100 mM β-mercaptoethanol). The cells were vortexed, placed on ice for 15 min, then centrifuged in an Eppendorf Microfuge for 20 min at 4°C at 1400 × *g* after adding an equal volume of phenol/water, 1/10th volume of chloroform-isoamyl alcohol, and 1/20th volume of 0.5 M Na acetate (pH 4.0).

Reverse transcription (RT) and polymerase chain reaction (PCR) analysis

RT was performed on total RNA (1 μg) using 5 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.-BRL) and 10 pmol of random primers. The mixture was heat-denatured for 5 min at 85°C. Total reaction volume was 20 μl in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, and 3 mM $MgCl_2$. PCR assays were performed in a total volume of 50 μl using all the RT product in a PCR buffer containing 100 mM Tris-HCl (pH 8.4), 500 mM KCl, 15 mM $MgCl_2$, 100 μg/ml bovine serum albumin, and 2 μM of each of the four deoxynucleoside triphosphates (Pharmacia), 10 pmol primer A (forward primer), 10 pmol primer B (reverse primer), and 1.0 U *Taq* DNA polymerase (Promega, Madison, Wis.). The PCR reactions involved an initial incubation at 95°C for 5 min and then annealing at 55°C for 1 min, extension at 72°C for 1 min, and denaturation at 95°C for 1.5 min. Thirty cycles of amplification were used. All PCR experiments included one control tube with no reverse transcription step.

PCR-amplified products were resolved in a 1.0% TBE agarose gel, transferred to nylon membranes, and validated by probing with ^{32}P-end-labeled oligonucleotide probes, which were used for PCR as described (27).

Quantification of the PCR products was performed using the Image-Quant PhosphorImager (Molecular Dynamics Technologies, Sunnyvale, Calif.) and normalized with a PCR-amplified housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequences of the oligonucleotides used in the RT-PCR analysis were as described (16): IL-1β, forward primer 5′-ATGGCAGAAGTACCTGAGCTG-3′; reverse primer 5′-TTCCTTGAGGCCCAAGGCCAC-3′; GAPDH forward primer 5′-CCATGGAGAAGGCTGGGG-3′; reverse primer 5′-CAAAGTTGTCATGGAGCC-3′. The semi-quantitative nature of our RT-PCR protocol, the precautions taken, and the controls used have all been described (28).

Electrophoretic mobility shift assays (EMSA)

To determine the NF-κB binding activity in the rgp350-treated and control cells, EMSA were performed. For this purpose, whole-cell extracts were prepared as described (21). Briefly, cells were pelleted by centrifugation 4 h after induction by various agents, washed with ice-cold PBS, and resuspended in 0.5 ml of the lysis buffer (20 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 10% glycerol, 10 mM sodium molybdate, 1 mM DTT) containing protease inhibitors: 0.5 mM PMSF and 1 μg/ml of each of pepstatin, leupeptin, and aprotinin (all inhibitors from Boehringer Mannheim, Laval, Québec). Cells were lysed by adding 2 M KCl dropwise to a final concentration of 0.5 M KCl, gently mixed by rotation at 4°C for 30 min, and centrifuged at 45,000 × *g* for 1 h at 4°C in a Beckman Ultracentrifuge. The supernatants were diluted to 0.1 M KCl with the lysis buffer and protein concentrations of the supernatants were determined using BCA protein assay, as described above.

For EMSA, double-stranded DNA oligonucleotides representing NF-κB sites in IL-1β promoter (−297 to −288, 5′-GGGAAAATCC-3′, IL-1β-κB), and a mutant version of the IL-1β-κB 5′-ACTAAATTCC-3′, which lacks NF-κB binding ability, were used. A double-stranded DNA oligonucleotide corresponding to the c-AMP response element (CRE) was also used in some assays; all these oligonucleotides have been

described earlier (21, 27, 29, 30). Five micrograms of the protein from whole-cell extracts were preincubated with 5 μg of poly (dI:dC) for 10 min at 4°C for reducing nonspecific binding to the oligonucleotides, then 20 ng of the ^{32}P-end-labeled oligonucleotides was added to the mixture and incubated for 20 min at room temperature. In competition assays, a 200-fold molar excess of the unlabeled oligonucleotide was added to the mixture during preincubation. For mobility supershifts, performed to identify the binding proteins to the oligonucleotides in EMSA, 1 μg of the p50-, p65-, or c-Fos-specific monoclonal antibodies was added during the preincubation period. After incubation, the samples were analyzed on a 6% native Tris-glycine PAGE, migrated at 150 V for 5 h, dried, and exposed to X-ray films for different lengths of time.

RESULTS

EBV rgp350 induces the production of IL-1β in human AMM

To see the effect of rgp350 on the production of IL-1β in AMM, 2×10^5 cells were incubated in 1 ml of the culture medium and rgp350 was added to these cultures to a final concentration of 100 ng/ml. As controls, cells were treated with infectious EBV, noninfectious UV-EBV, or TPA (25 ng/ml). The concentrations of IL-1β were determined in the culture supernatants (secreted form) and cytosolic preparations (cytosolic form) 36 h later and are depicted in **Fig. 1**. A low-level constitutive production of both forms of this cytokine was observed in these experiments. The presence of rgp350 in the cultures caused a marked increase in both the secreted and cytosolic forms of the cytokine. UV-EBV also stimulated the production of both forms of IL-1β. However, in marked contrast to the UV-EBV and rgp350, infectious EBV lacked this ability; virtually no differences were observed in the secreted and cytosolic forms of IL-1β between infectious EBV-treated and untreated control AMM cultures. Pretreatment of rgp350 with gp350-specific 72A1, but not with 2L10 mAbs, markedly inhibited this enhanced production of both forms of this cytokine (Fig. 1; compare lanes 3, 5, and 6). It is noteworthy that mAb 72A1 blocks binding of gp350 to CR2/CD21 whereas mAb 2L10 has no significant effect on this binding (6, 31). These results suggest that the binding of rgp350 to the EBV receptor CR2/CD21 on AMM was the specific event that caused the stimulation of IL-1β production (also see below). The cytosolic IL-1β concentration was always higher (~twofold) than its secreted form in AMM whether or not these cells were induced.

Effect of CR2-specific mAb OKB7 on rgp350-mediated stimulation of IL-1β production

The results reported above with 2L10- or 72A1-pretreated rgp350 suggest that the binding of this

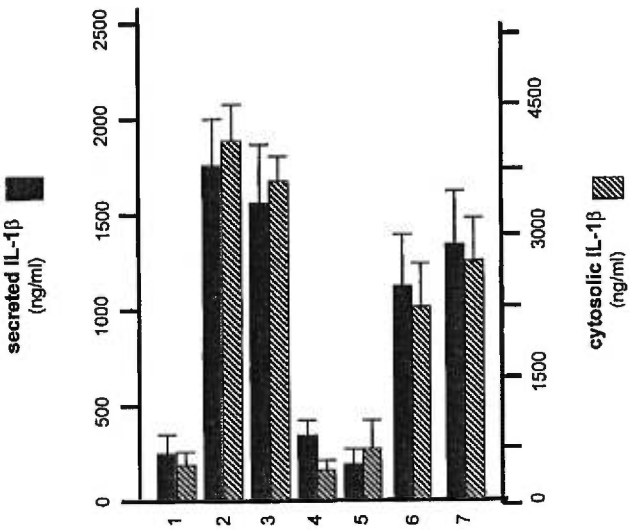

Figure 1. Production of IL-1β protein in rgp350-treated monocyte-macrophages. Plastic-adherent monocyte-macrophages (2×10^5/ml) were cultured in the wells of a 24-well culture plate in the presence of TPA (25 ng/ml), rgp350 (100 ng/ml), or infected with EBV or UV-EBV. Thirty-six hours later, cell-free culture supernatants were collected and cells were harvested for cytosolic preparations. The concentration of IL-1β was determined in the supernatants (secreted form; see scale at the left of the figure) and cytosolic preparations (see scale at the right of the figure) using a commercial ELISA Kit. Average concentrations of IL-1β ± SE are shown from three replicate cultures: column 1, untreated cells; column 2, TPA-treated; column 3, rgp350-treated; column 4, EBV-infected; column 5, rgp350-pretreated with mAb 72A1; column 6, rgp350-pretreated with mAb 2L10; column 7, UV-inactivated EBV.

glycoprotein to CR2/CD21 stimulated the production of IL-1β. To confirm that this binding was necessary for the stimulated production of IL-1β, we pretreated 2×10^5 AMM by adding CR2/CD21-specific mAb OKB7 to the culture medium (10 μg/ml final concentration) 30 min before addition of rgp350. This mAb blocks the binding of gp350 and EBV to CR2/CD21 on target cells (5). As shown in **Fig. 2**, pretreatment of cells with OKB7 markedly inhibited the rgp350-induced synthesis of the secreted (~50% reduction) and cytosolic (~75% reduction) forms of IL-1β. The treatment of AMM with OKB7 alone did not have a significant effect on the constitutive synthesis of the two forms of this cytokine. These results clearly indicate that the rgp350-induced stimulation of IL-1β production in AMM is dependent on the specific binding of this glycoprotein to the EBV receptor CR2/CD21.

Effect of rgp350 on IL-1β mRNA

To examine whether the rgp350-induced production of IL-1β from AMM was accompanied by increased steady-state levels of IL-1β mRNA in these cells, 2×10^5 cells were incubated in 1 ml of the culture

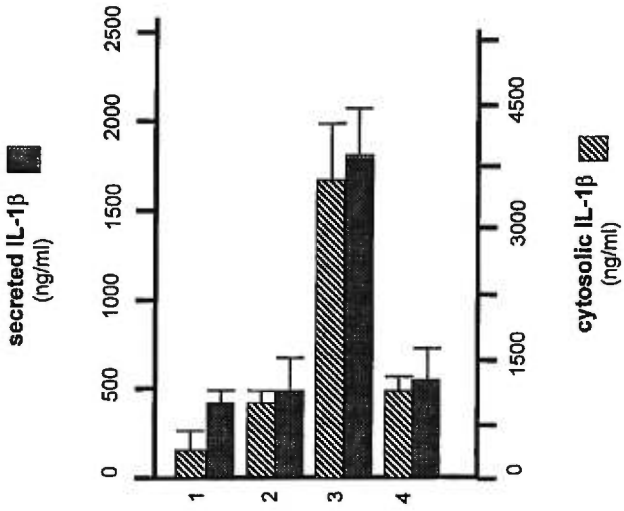

Figure 2. Production of IL-1β in rgp350-treated monocyte-macrophages after pretreatment with mAb OKB7. The cells were cultured and stimulated with rgp350 as described in the legend to Fig. 1. However, these cells were also pretreated with mAb OKB7 (10 μg/ml) for 30 min at room temperature before the addition of rgp350. The concentration of IL-1β in the culture supernatants as well as in the cytosolic preparations was determined as described in Fig. 1. Shown here are the average IL-1β concentrations ± SE from three replicate cultures. Column 1: untreated cells; column 2: cells treated with OKB7 mAb; column 3: cells treated with rgp350; and column 4: cells pretreated with OKB7 mAb, followed by rgp350.

medium and treated with rgp350 (with or without prior treatment with OKB7), or rgp350 that was pretreated with 2L10, 72A1, or control antibodies at 37°C as described above. After 6 h of incubation, the cells were collected and processed for determination of IL-1β mRNA by the RT-PCR method as described in Materials and Methods. As shown in **Fig. 3*A***, pretreatment of cells with OKB7 and pretreatment of rgp350 with 72A1 caused a marked reduction in the induction of IL-1β mRNA expression.

A kinetic study of IL-1β mRNA induction by rgp350 was undertaken. Figure 3*B* shows the relative levels of IL-1β mRNA in rgp350-treated AMM (100 ng of rgp350 per 2×10^5 AMM/ml of the medium) at different time points after the treatment. IL-1β mRNA was induced as early as 1 h after adding rgp350 to the cultures and reached a peak level at 4–6 h post-treatment. Since UV-EBV behaved essentially like rgp350 in inducing the synthesis of IL-1β in AMM, we repeated this kinetic experiment using UV-EBV. As shown in Fig. 3*C*, UV-EBV also induced IL-1β mRNA in AMM by 1 h; however, the levels tended to decline earlier.

Although AMM constitutively express low basal levels of secreted and cytosolic forms of IL-1β, in our hands no signal for IL-1β mRNA was visible in untreated cells in the two autoradiograms shown here. This was simply due to shorter exposure times of these blots to X-ray films, since upon prolonged exposure IL-1β messages were detected in untreated cells. These exposures, however, caused over-darkening and intermingling of signals from treated cells (data not shown). These results clearly demonstrate that treatment of human AMM with rgp350 or UV-EBV causes a rapid increase in the steady-state level of IL-1β mRNA; furthermore, this increase in IL-1β is induced by specific binding of gp350 to CR2/CD21 on these cells.

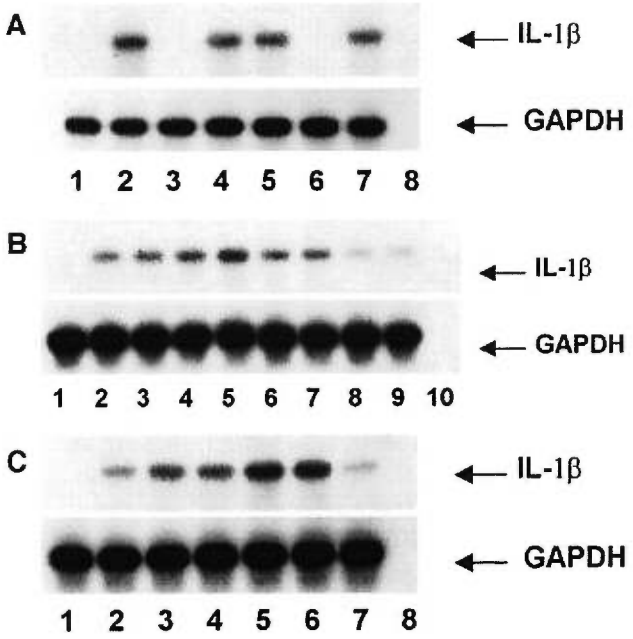

Figure 3. *A)* RT-PCR analysis of IL-1β mRNA in differentially treated monocyte-macrophages. Adherent monocyte-macrophages (2×10^5/ml) were treated as indicated; 6 h post-treatment, the cells were harvested to determine mRNA for IL-1β and GAPDH by RT-PCR as described in Materials and Methods. Lane 1: untreated cells; lane 2: rgp350 treated; lane 3: rgp350 preincubated with mAb 72A1; lane 4: rgp350 preincubated with mAb 2L10; lane 5: rgp350 preincubated with heat-denatured 72A1; lane 6: cells preincubated with mAb OKB7, followed by treatment with rgp350; lane 7: cells preincubated with heat denatured OKB7, followed by rgp350 treatment; lane 8: non-RT control. *B)* Time course of IL-1β gene activation by rgp350. 2×10^5 AMM were cultured in 1 ml of the culture medium with or without the addition of rgp350 (100 ng/ml). Cells were harvested at the indicated times, and IL-1β and GAPDH mRNAs were analyzed by RT-PCR. Lane 1: untreated cells; lane 2: 30 min; lane 3: 60 min; lane 4: 2 h; lane 5: 4 h; lane 6: 6 h lane 7: 8 h; lane 8: 10 h; lane 9: 12 h; and lane 10: a non-RT control. *C)* Time course of IL-1β gene activation by UV-EBV treated AMM cells. 2×10^5 AMM were incubated with 100 μl of the UV-inactivated EBV or with an equal volume of mock infection fluid for 1 h at 37°C and then cultured in 1 ml of culture medium. The cells were harvested at the indicated time points after the start of the cultures and processed to determine IL-1β and GAPDH mRNAs by RT-PCR. Lane 1: untreated cells; lane 2: 60 min; lane 3: 2 h; lane 4: 4 h; lane 5: 6 h; lane 6: 8 h; lane 7: 12 h; and lane 8: non-RT control.

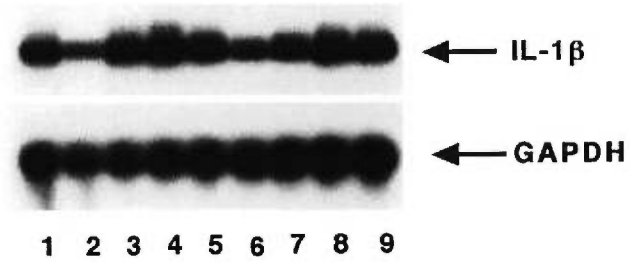

Figure 4. IL-1β gene transcription in rgp350-treated monocyte-macrophages in the presence of inhibitors of different enzymes. 2×10^5 monocyte-macrophages were cultured in the presence of rgp350 (100 ng/ml) with or without the presence of specific inhibitors of different enzymes involved in signal transduction pathways. The cells were harvested 6 h after the start of the cultures and processed to determine IL-1β and GAPDH mRNAs by RT-PCR. The lanes show mRNA from cells treated with 1: rgp350; 2: infectious EBV; 3: UV-EBV; 4: TPA; 5: rgp350 + staurosporin; 6: rgp350 + bisindoylmaleimide; 7: rgp350 + LY294002; 8: rgp350 + tyrphostin; 9: rgp350 + MDL-12,330A-HCl. Since data from Fig. 3*A-C* show negligible basal IL-1β mRNA expression, an additional control for untreated cells was not included in this figure.

The signal-transduction pathways involved in rgp350-mediated induction of IL-1β production

It has been suggested that because of its short cytoplasmic tail, CR2/CD21 is unable to transduce signals intracellularly (32). However, the data presented above clearly indicate that upon binding to rgp350 or UV-EBV, CR2/CD21 can transduce signals that result in the induction of IL-1β synthesis. To determine the nature of the signal transduction pathway(s) used by CD21/CR2 in this IL-1β induction, we treated AMM with rgp350 (100 ng/ml) with or without the presence of reagents that inhibit activities of various kinases. These reagents included specific and nonspecific inhibitors for adenyl cyclase, PKC, PI3-K, and protein tyrosine kinases. These inhibitors and the concentrations used are provided in Materials and Methods. The cells were incubated at 37°C for 6 h and the quantity of IL-1β mRNA was determined by RT-PCR, as described above. The results are depicted in **Fig. 4** and **Table 1** and demonstrate that the induction of IL-1β mRNA was markedly reduced when cells were stimulated with rgp350 in the presence of specific inhibitors for PKC (80% reduction, lane 6 in Fig. 4) and PI3-K (50% reduction, lane 7 in Fig. 4). Even when used at higher concentrations (e.g., up to 50 nM for staurosporin), only PKC and PI3-K inhibitors blocked the induction of IL-1β mRNA; other inhibitors had no significant effect (data not shown).

Activation of NF-κB in rgp350-treated cells

A rapid induction of IL-1β mRNA in AMM after treatment with rgp350 suggested the involvement of rapidly inducible transcription factors (e.g., NF-κB) in this process. Furthermore, IL-1β is a proinflammatory cytokine and constitutive, chronic activation of NF-κB in inflammatory conditions is well known (reviewed in refs 33–35). These considerations and the fact that three NF-κB binding sites have been demonstrated in the promoter region of IL-1β gene (28, 29, 36) prompted us to investigate whether rgp350 treatment induces NF-κB binding activity in human AMM. Therefore, we conducted EMSA assays using double-stranded DNA oligonucleotides representing NF-κB binding sites in the IL-1β as well as a mutant site (detailed in Materials and Methods). As shown in **Fig. 5*A***, whole-cell extracts from untreated AMM showed constitutive NF-κB binding activity (consistent with the low-level synthesis of this cytokine in these cells) that was markedly increased (~8.0-fold) on treatment with rgp350. The specificity of the NF-κB binding in EMSA was determined by cold competition with the mutant site and with unlabeled IL-1β-κB oligonucleotide. Addition of the cold IL-1β-κB to the assays abrogated binding to the oligonucleotide probe, whereas competition with the mutant IL-1β-κB did not cause this reduction (Fig. 5*A*, lanes 5, **Fig. 6**). In addition to NF-κB, the IL-1β promoter region also contains multiple CREB (cyclic AMP-responsive element binding protein) sites. Our results using gp350 or EBV showed no enhanced CREB binding (data not shown).

To confirm that the complexes bound to the IL-1β-κB oligonucleotides from the whole-cell extracts of rgp350-treated AMM contained authentic transcription factors belonging to the NF-κB/Rel family, we added p50-, p65,- or c-Fos-specific mAbs (1 μg/reaction) during preincubation with poly (dI:dC) and performed the EMSA. The p65 (RelA) and p50 are classical prototypic members of the NF-κB/Rel family whereas c-Fos is a component of another inducible

TABLE 1. *Densitometric analysis of IL-1β RT-PCR products in differentially treated AMM cells*[a]

Lane	1	2	3	4	5	6	7	8	9
Relative change	—	70% ↓	7% ↓	24% ↓	5% ↓	62% ↓	18% ↓	9% ↓	3% ↓

[a] The IL-1β RT-PCR products were quantitated by laser densitometry and normalized to GAPDH levels (described in Materials and Methods). The relative increase/decrease of IL-1β PCR product for each cell treatment compared to the basal level (Fig. 4, lane 1) was calculated and is shown here. The lanes represent treatments as shown in legend to Fig. 4.

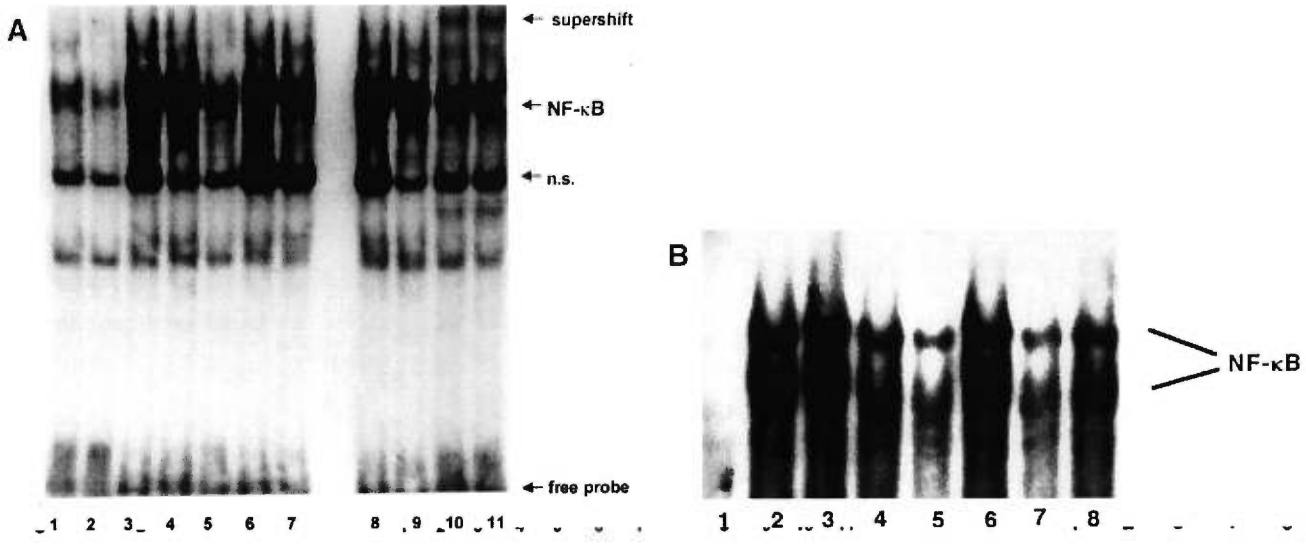

Figure 5. *A)* Activation of NF-κB in rgp350-treated monocyte-macrophages. Plastic-adherent monocyte-macrophages were treated as indicated in each lane and whole-cell extracts (WCE) were prepared 4 h after the treatment. Activation of NF-κB was determined by EMSA using 5 μg of the WCE and oligonucleotides containing κB sites as described in Materials and Methods. Lane 1: unstimulated cells; lane 2: EBV-infected cells; lane 3: rgp350-induced; lane 4: UV-EBV-induced; lane 5: competition with IL-1β κB; lane 6: competition with mutant IL-1β κB; lane 7: competition with CRE; lane 8: rgp350-treated cells; lane 9: supershift with anti-c-Fos antibodies; lane 10: supershift with anti-p50 antibodies; lane 11: supershift with anti-p65 antibodies. *B)* Inhibition of gp350-mediated activation of NF-κB by gp350-specific antibodies and specific inhibitors of enzymes. Human peripheral blood monocyte-macrophages were stimulated with rgp350. In some cases the cells were also treated with gp350-specific monoclonal antibodies (72A1 or 2L10) or specific inhibitors of enzymes; activation of NF-κB in the cells was determined by EMSA using 5 μg of the whole-cell extracts, as described in Materials and Methods. Lane 1: unstimulated cells; 2: cells treated with TPA; 3: cells treated with rgp350; 4: cells treated with 2L10 mAb plus rgp350; 5: cells treated with 72A1 mAb plus rgp350; 6: cells treated with staurosporin plus rgp350; 7: cells treated with LY294002 plus rgp350; 8: cells treated with bisindoylmaleimide plus rgp350.

transcription factor, activation protein-1 (AP-1; refs 33, 35). As shown in Fig. 5*A*, the IL-1β-κB bound complexes were supershifted when anti-p50 or anti-p65 antibodies were added but not when anti-c-Fos-antibodies were added. The relative strength of the supershift was considerably greater with anti-p50 antibody than with anti-p65 antibody. These results suggest that treatment of AMM with rgp350 induces the activation of p50 and p65 in these cells.

Finally, we determined whether gp350-specific monoclonal antibodies and PI3-K and PKC inhibitors that inhibit rgp350-induced activation of IL-1β gene also inhibit rgp350-mediated NF-κB activation in human monocyte-macrophages. We pretreated these cell cultures with these regents, treated them with rgp350, and determined NF-κB activation by EMSA (see Materials and Methods). As shown in Fig. 5*B*, 72A1 mAb and specific inhibitors of PKC and PI3-K significantly inhibited this activation in these cells, which is consistent with our data on the inhibition of IL-1β gene activation by these reagents.

Inhibition of rgp350-mediated NF-κB activation inhibits the activation of the IL-1β gene

To find out whether rgp350-mediated activation of NF-κB is essential for the activation of the IL-1β gene, the AMM were incubated with this glycoprotein in the presence of a cell-permeable synthetic peptide SN50 that has been shown to prevent the nuclear translocation of at least three members of the NF-κB family (p50, p65, and c-Rel) in human monocytic cells (22). This is tantamount to inhibition of the activation of these factors, since without their translocation to the cell nucleus they cannot bind to the target DNA sequences and mediate their effects. As shown in Fig. 6, the inhibitor peptide not only significantly reduced the production of IL-1β in the culture supernatants, but also decreased the steady-state levels of IL-1β mRNA as compared to the rgp350-treated cells. The control peptide had no significant effect on IL-1β gene activation in rgp350-treated cells, suggesting that the activation of NF-κB in rgp350-treated cells was a prerequisite for IL-1β gene expression.

DISCUSSION

The results presented here show that the major EBV envelope glycoprotein gp350 induces the production of IL-1β in human monocyte-macrophage cultures. In this capacity, rgp350 is comparable to the phorbol ester TPA. In contradistinction to rgp350, EBV did not induce this cytokine in these cells, whereas noninfectious UV-EBV behaved like rgp350.

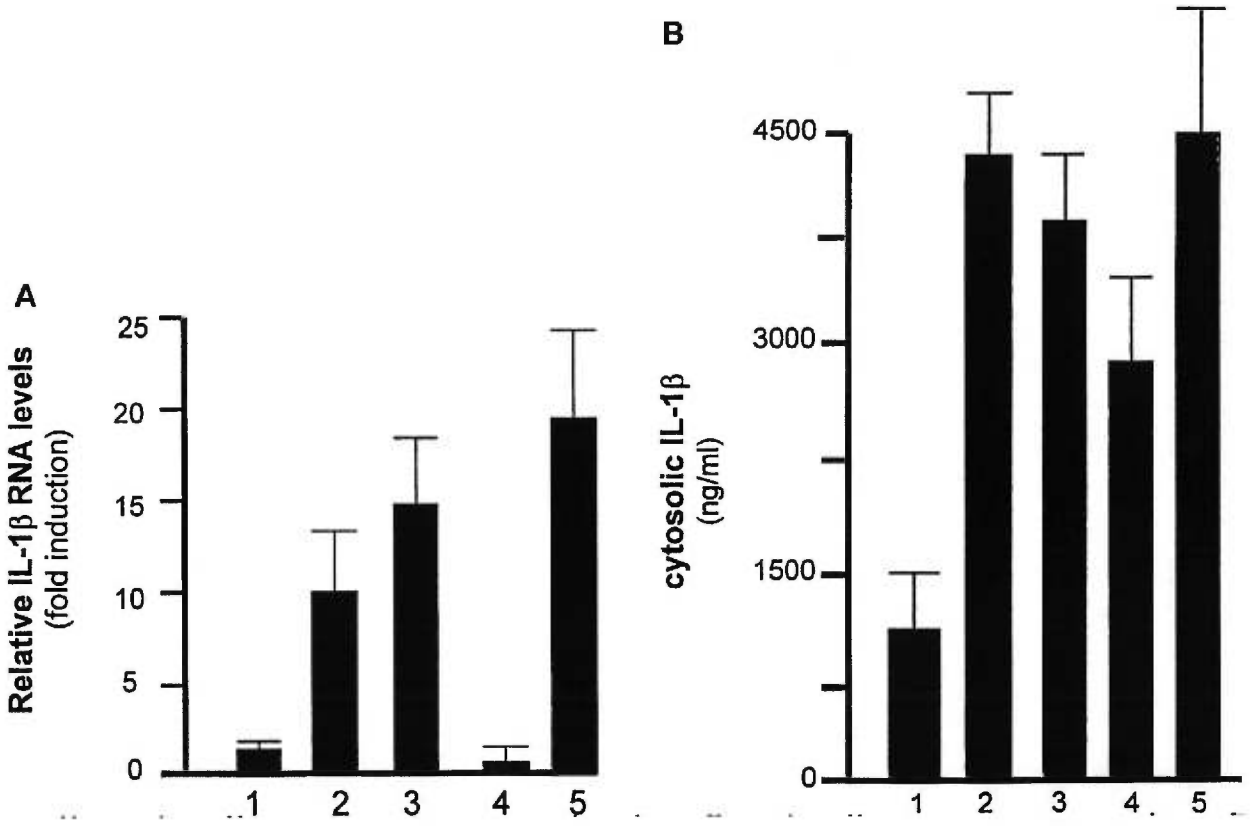

Figure 6. The effect of NF-κB inhibitor peptide SN50 on the rgp350-mediated induction of IL-1β gene in monocyte-macrophages. The plastic-adherent monocyte-macrophages were incubated with rgp350 (100 ng/ml) in the presence of the inhibitor (SN50) or control (SN50M) peptide (20 μg/ml). Cells were harvested 6 h later for IL-1β and GAPDH mRNA determinations by RT-PCR (described in Materials and Methods). The cytosolic fractions were prepared 36 h later and analyzed for IL-1β content by a commercial ELISA kit. *A)* Results from the RT-PCR analysis; *B)* average IL-1β concentrations from three replicate cultures. The Y-bars represent SD; lane 1: untreated cells, 2: rgp350-treated cells; 3: rgp350-treated cells in the presence of κB control peptide, 4: rgp350-treated cells in the presence of the κB inhibitor peptide; 5: TPA-treated cells positive control).

Experiments with rgp350- and CR2-specific mAbs unequivocally demonstrated that the effect of gp350 on the activation of IL-1β gene in human monocyte-macrophages was dependent on its interaction with CD21/CR2. Previously we demonstrated that EBV is capable of modulating the expression of cytokine genes in human PBMC (12, 16, 17). Using unfractionated PBMC, we had shown that EBV remarkably down-regulated TNF-α production in these cells whereas its effects on IL-1β production were slightly stimulatory (12, 16, 17).

The differences in the results of these and our previous studies (12, 16, 17) may be explained by the fact that earlier we had used whole, unfractionated PBMC whereas in the present study we used purified monocyte-macrophage cultures. It is quite possible that one or more subpopulations of PBMC may be responding to EBV in a different way than monocyte-macrophages with respect to the activation of IL-1β gene. Gp350 has recently been shown to induce the production of IL-6 in human B cells (18); however, to our knowledge this the first report implicating this glycoprotein in the induction of proinflammatory cytokines from human monocyte-macrophages. In addition, whereas gp350 strongly induces IL-1β mRNA and protein, EBV appears to slightly increase IL-1β RNA production while not enhancing protein expression; this suggests the existence of some form of post-transcriptional regulation. We and others earlier demonstrated that human monocytic cells express CR2 (12, 14), although these cells are not known as being EBV targets *in vivo*. It is conceivable that other CR2-expressing cell types (e.g., T and follicular dendritic cells) may also be affected by the immune-modulating properties of EBV and gp350. We have incubated infectious EBV with AMM cells for up to 7 days, but were not able to show EBV RNA or protein from these cells, suggesting that this virus may simply interact with their cell membrane receptors without undergoing replication (unpublished observations). It has been shown that EBV interacts with CR2/CD21-expressing thymocytes and interferes with the thymic selection process by inducing IL-2 from them (37). Furthermore, we recently demonstrated that EBV causes the release of TGF-β from human platelets by binding to its receptor on these blood elements (38).

The binding of gp350 to CR2/CD21 on target cells

is the first step in the EBV infection process and the production of cytokines like IL-1β (which could induce a strong anti-viral immune and inflammatory response in the infected host) may not be desirable from the virus point of view. Thus, EBV has devised strategies to block this response. These strategies may be in the form of a component(s) of the viral particle that suppresses this response but is somehow inactivated in the UV-irradiated virus. In an alternate and more likely (but not mutually exclusive with the preceding) scenario, infectious EBV may induce the expression of one or more viral and/or cellular genes immediately after infection, which prevents the production of gp350-mediated cytokines. One obvious viral candidate gene for this effect is the viral homologue of human IL-10 gene (BCRF1 or vIL-10; 1, 8), which is comparable to hIL-10 in its capacity to inhibit cytokine induction (39, 40). We are currently investigating the role of vIL-10 in the down-regulation of gp350-induced IL-1β production.

The present results demonstrate that rgp350 induces the activation of NF-κB or the Rel family of transcription factors in a PI3-K- and PKC-dependent manner. These transcription factors, which occur as inactive homo and/or heterodimers in the cytoplasm due to their binding with inhibitory proteins IκB, can be rapidly activated by a wide variety of stimuli (LPS, PMA, IL-1β, TNF-α, etc.; reviewed in refs 33, 35). These stimuli induce phosphorylation at specific serine/threonine sites of IκB, which then become polyubiquinated and degraded via proteasomes. The degradation of classical IκB unmasks the nuclear localization signals on NF-κB dimers, which then migrate to nucleus and bind to specific response elements—NF-κB binding sites. These sites exist in the promoter regions of numerous genes involved in cellular growth, differentiation, and inflammatory and immune responses. Three such sites have been found in the regulatory region of the IL-1β gene, and NF-κB activation is known to induce IL-1β gene expression (41, 42). These sites also exist in viral promoters, e.g., HIV-I LTR. IL-1β enhances HIV-1 replication through activation of NF-κB, which bind to NF-κB binding sites in the viral LTR (reviewed in ref 33). Activation of NF-κB may also be needed for a successful EBV infection. It is noteworthy that EBV infects resting B cells, which have little or no constitutively activated NF-κB. Sugano et al. (43) showed that EBV activates this family of transcription factors via CR2/CD21 in tonsillar B cells. This activation is PKC dependent and is needed to drive transcription from the Wp promoter. Wp is the initial EBV latent gene promoter located in the major long terminal repeat (BH1W; ref 1); EBNA2 and EBNA leader proteins are initially transcribed from this promoter. Our results indicate that rgp350 can also activate these transcription factors in human monocyte-macrophages via PKC and PI3-K. Further work will be needed to know whether this activation involves induced phosphorylation and degradation of IκB, increased production of the transcription factors, or affects the activities of PP2A or of the newly discovered IκB kinases.

The EBV receptor (CR2) is a 140 kDa type II integral membrane protein and belongs to the regulators of complement activation gene family (reviewed in refs 2, 9). It is an important member of the B cell antigen receptor complex and can dramatically augment the immune response to an antigen if the latter is bound to the natural ligand of CR2, i.e., C3dg (9). It occurs singly as well as in association with CD19, TAPA-1 (target for anti-proliferation antibody-1), and CD35 (reviewed in refs 2, 9). It uses CD19 and/or TAPA-1 and transduces signals via phospholipase C and PI3-K. Activation of B cells causes phosphorylation of CR2, cross-linking of CR2 by extracellular ligands, including EBV on B cells, and increases their proliferation. C3d or a 16 amino acid peptide corresponding to the CR2 binding domain of C3d has also been shown to phosphorylate pp105 in CR2-positive cells (44). CR2 can also interact with tumor suppressor protein p53, a Ca^{2+} binding protein p68, and with ribonucleoprotein p120 (45). It has recently been shown that binding of EBV to CD21/CR2 on B-lymphocytes activates PI-3-K independent of CD19 (46); these results, along with our previous findings demonstrating a lack of CD19, suggest that the presence of CD21 on AMM cells may be sufficient to transduce signals through the cell membrane, resulting in cytokine gene activation. Our results also suggest involvement of the PI-3-K pathway for cellular activation, in agreement with the results shown in B-lymphocytes (46). Although CR2 occurs on monocytic cells, these cells are devoid of CD19 (2). Our results suggest that despite the lack of CD19, rgp350-dependent stimulation of CR2 in these cells activates NF-κB in addition to PI-3K and PKC (see below).

A wide variety of extracellular stimuli can cause activation of NF-κB. Chronic inflammatory conditions are usually accompanied by the constitutive high-level activation of these factors. Viral transforming proteins, e.g., Tax of HTLV-1 and LMP-1 of EBV activate NF-κB (47, 48). It has been shown that activated NF-κB prevents cellular apoptosis (49, 50). Activation of these transcription factors by these viral proteins may be important for their cell transforming ability. The present study shows for the first time the involvement of a nontransforming envelope glycoprotein of EBV (i.e., gp350) in the activation of these factors in human monocyte-macrophages. Hemagglutinin of influenza virus, another virus that infects quiescent cells, is known to activate these transcription factors (51). Thus, it appears that vi-

ruses that infect quiescent cells have evolved the strategy to activate NF-κB or other transcription factors at the beginning of their infection process.

The data presented here also show that rgp350-induced activation of NF-κB in human monocyte-macrophages is PKC and PI3-K dependent. PKC is a cytosolic serine/threonine kinase that upon activation is translocated to the cell membrane (reviewed in ref 52). It has been shown that gp350 induces IL-6 production in human B cells via activation of PKC (18). Our results suggest that this glycoprotein can also activate this kinase in human monocyte-macrophages. Whether PCK activation in gp350-stimulated AMM also results in the activation of transcription factors other than NF-κB is not known. Our unpublished data suggest that transcription factors AP-1, CREB, and STAT-3 are not activated in human monocyte-macrophages by this glycoprotein.

PI3-K catalyzes the synthesis of second messengers phosphatidylinositol-3,4 biphosphates and -3, 4, 5 triphosphates (reviewed in ref 53). This is an important serine/threonine kinase that is involved in diverse processes such as transformation, inflammation, cell growth, etc., and is activated by gp350. One of the most important efferent functions of P13-K is the activation of cellular AKT, a homologue of viral oncogene AKT that is known to prevent apoptosis (53). Recently, PI3-K itself has been shown to have transforming ability (54). In keeping with the ability of gp350 to activate NF-κB and PI3-K, it is tempting to speculate a role for gp350 in apoptosis and cell survival.

Because of the potent and widespread inflammatory effects in the human body, the production and activities of IL-1β are tightly regulated at multiple steps: at transcription, mRNA stability, translation, processing of the protein, and secretion (reviewed in ref 20). Our work shows an increase in the steady-state levels of IL-1β mRNA in rgp350-treated cells, which may be due to increased mRNA stability and/or increased transcription. More important, the increased mRNA levels result in increased production of IL-1β protein. gp350 differs in this respect from certain other biological inducers of IL-1β—e.g., C5a, which causes an increase in IL-1β mRNA but no increase in IL-1β protein production (55). Further work is required to determine whether gp350 modulates IL-1β production at other steps of its regulatory mechanism.

The rgp350-mediated stimulation of IL-1β production in human monocyte-macrophages reported here has implications for EBV biology not only in terms of virus–cell interactions, but also for its immunology and vaccinology. Gp350 is a target protein for anti-EBV cellular and humoral immunity. It is expressed most abundantly on the virion and on the surfaces of productively infected cells. Gp350-based vaccines have proved effective in simian models in protecting animals from EBV-induced fatal lymphomas (56). By inducing the secretion of proinflammatory cytokines, the gp350 itself may be acting as an adjuvant. FJ

We thank the Medical Research Council of Canada and the J.-L. Lévesque Foundation for support. We are grateful to Dr. A. D. Morgan for rgp350, Dr. G. R. Pearson for 2L10, Dr. Jean Gosselin for 72A1 mAb, Dr. G. Ahronheim for comments on this manuscript, and Micheline Patenaude for excellent secretarial assistance.

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Received for publication February 22, 1999.
Revised for publication July 8, 1999 .

Binding of the Epstein-Barr Virus Major Envelope Glycoprotein gp350 Results in the Upregulation of the TNF-α Gene Expression in Monocytic Cells via NF-κB Involving PKC, PI3-K and tyrosine kinases.1

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Running title: Effects of EBV gp350 on TNF-α gene expression in monocytic cells

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1 We thank the Medical Research Council of Canada and the J-Louis Lévesque Foundation for support.

ABSTRACT

Epstein-Barr virus (EBV) is a human herpesvirus that interacts with various immunocompetent cells that carry the EBV receptor (CD21/CR2). EBV binds to CR2 through its major envelope glycoprotein 350 (gp350). Previously we had demonstrated that EBV and other human herpesviruses are capable of modulating cytokine synthesis through the deregulated expression of cytokine genes interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interleukin-2 (IL-2). Here we show that, in contrast to infectious EBV, purified recombinant gp350 upregulates TNF-α gene expression in human monocyte/macrophages (M/M) as well as in a monocytoid cell line, U937. Our results also demonstrate that this increased expression is due to both enhanced transcription and stability of TNF-α mRNA in gp350-treated cells. The specificity of this effect is evidenced by the fact that pre-incubation of cells with anti-CR2 monoclonal antibody OKB7, which blocks binding of gp350 to CR2, inhibits the above mentioned effects of gp350. Furthermore, we demonstrate that activation of TNF-α by gp350 is mediated by NF-κB through signal transduction pathways involving PKC, PI3-K and tyrosine kinases. To our knowledge this is the first report describing the modulation of TNF-α gene expression by the EBV-gp350 molecule following its interaction with the viral receptor CR2 on cells of the monocytic lineage.

INTRODUCTION

Epstein-Barr virus (EBV) is a ubiquitous human herpes-virus whose genome has been found in various lymphoid and epithelial tumors (Alero-Thomas *et al.*, 1991; Neidobitek & Young, 1994). Primary EBV infection in adolescents generally causes glandular fever or infectious mononucleosis, a self-limiting lymphoproliferative disorder. By adulthood more than 90% of individuals become infected and remain healthy life long carriers of the virus while some develop EBV-associated diseases including African Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's and non-Hodgkin's lymphoma (Banks & Rouse, 1992). Viral infection persists in B-lymphocytes mainly in latent form. In vitro, EBV can transform and immortalize human B cells into continuously growing lymphoid cell lines (LCL). The infection of B cells is mediated by the type II complement receptor, CR2, whose natural ligand is the C3d fragment of complement (Fingeroth *et al.*, 1984; Frade *et al.*, 1985; Nemerow *et al.*, 1985). CR2 is a member of the B cell antigen receptor complex and its ligands (including EBV) can modulate B cell functions (Cambier *et al.*, 1994). The major envelope glycoprotein complex of EBV-gp350/220 (gp350) binds to CR2 and initiates the infection process (Fingeroth *et al.*, 1984; Frade *et al.*, 1985; Nemerow *et al.*, 1985; Tanner *et al.*, 1987). CR2 occurs on many human cell types other than B cells, i.e.: follicular dendritic and endothelial cells, thymocytes, T cells, epithelial cells, and monocytes (Fingeroth *et al.*, 1988; Hedrick *et al.*, 1994; Inada *et al.*, 1983; Jones *et al.*, 1995; Kaufman-Patterson *et al.*, 1995a, 1995b; Sauvageau *et al.*, 1990). Thus, EBV may potentially interact with these cell lines via CR2 and modulate their functional activities. Infection of immature thymocytes by EBV induces cellular proliferation and may predispose the host to autoimmune disorders by avoiding negative T-cell selection (Kaufman-Patterson *et al.*, 1995a, 1995b; Todd & Tsoukas, 1996).

Acute and chronic EBV infections are accompanied by immune suppression and cytokine deregulation (Gosselin *et al.*, 1991, 1992a, 1992b). It has been found that the

binding of EBV or gp350 to B cells or LCL causes the secretion of interleukin-6 (IL-6) through a protein kinase C (PKC) dependent pathway (Tanner *et al.*, 1996). Our earlier studies suggested that EBV can modulate the secretion of TNF-α, IL-1, and IL-6 in human peripheral blood mononuclear cells (PBMC) as well as in cells of the monocyte/macrophage (M/M) lineage (Gosselin *et al.*, 1991, 1992a, 1992b). Kinetics of this cytokine deregulation suggested that binding of EBV to the cells via gp350 was sufficient to induce this deregulation. Gp350 not only contributes to the initiation of EBV infection but is also the target antigen for anti-EBV cellular and humoral immune responses (reviewed in Kieff, 1996). When cottontop tamarins (an animal model for EBV-induced B lymphomas) were immunized with gp350 alone, they were protected from EBV-induced tumors (Morgan *et al.*, 1988, 1993). Considering the potential of gp350 to modulate cytokine synthesis and therefore to provide insight into the immune modulation in EBV infections, we investigated the effects of the interaction of gp350 with cells of the M/M lineage, with regard to TNF-α gene expression. We also examined the effects of gp350 on selective signal transduction pathways and transcription factor complexes in these cells.

RESULTS

EBV gp350 activates TNF-α gene transcription and translation.

In initial experiments, we incubated AMM with different concentrations of the purified gp350 to determine the optimum concentration necessary for TNF-α gene stimulation. These experiments indicated that the optimal concentration of EBV gp350 necessary for maximal TNF-α transcriptional induction was 80-100 ng/ml. The results of a representative experiment are shown in Fig. 1A. Above 80-100 ng/ml, additional transcriptional activation of TNF-α was not significantly induced by gp350. Over the entire range of gp350 concentrations used, no increase in the induction of the housekeeping GAPDH gene was observed (Fig. 1A). Therefore, all subsequent experiments were carried out using 100 ng/ml of EBV-gp350. A time course study indicated that by 6 hr, a 14-fold increase in the transcription of TNF-α occurred after treatment with gp350 (Fig. 1B and C). The levels of TNF-α transcripts were normalized to GAPDH levels, and as the radiographs show, increased transcriptional activation did not occur beyond 6 hr (lane 4). Interestingly, this time course experiment demonstrated that at 24 hr TNF-α RNA was still present at levels 7.5 fold greater than baseline (Fig. 1B lane 10). Extension of this time course to 36 and 48 hr still demonstrated elevated levels, 5.3 fold and 3.6 fold, respectively, of TNF-α that diminished only after 48 hr (data not shown). Figure 1B, lane 11 shows a non-RT control PCR reaction indicating no DNA contamination during the RNA purification procedure.

We next examined the effect of gp350 vis a vis EBV on the TNF-α gene activation in AMM cells and the densitometric analysis of the RT-PCR products is shown in Table 1. TNF-α mRNA was detected by RT-PCR in AMM cells without any treatment and was enhanced 3.1 fold by gp350 at 100 ng/ml (Table 1, lane 4). Lane 2 confirmed our previous result that infectious EBV is capable of inhibiting TNF-α gene expression (Gosselin *et al.*, 1991). Interestingly, UV- and heat-inactivated EBV-treated cultures showed stimulatory activity (lanes 6 and 7). Lanes 8 and 9 demonstrate that inhibition of gp350 interaction with CR2 by pretreatment with CR2-specific OKB7 mAb significantly abrogated gp350 stimulatory activity (compare lane 4 with lanes 8 and 9). To further demonstrate gp350 specificity, cells pretreated with a gp350 specific mAb (2L10), which does not inhibit binding of gp350 to CR2, did not block the gp350-induced transcription of TNF-α messages (data not

shown). Control experiments using isotype-matched immunoglobins specific for 2L10 and OKB7 did not show any effect on TNF-α gene activation (data not shown)

To examine the effects of gp350 treatment on the expression of TNF-α mRNA in U937 cells, Northern blot analysis was performed and the densitometric results are shown in Table 2. We had previously shown that addition of EBV to cultured U937 cells resulted in the decrease in TNF-α expression and that up to 24 % of these cells express CR2 as determined by the binding of OKB7 (Gosselin *et al.*, 1991). Our results indicate that gp350, like PMA, is capable of stimulating TNF-α transcription (Table 2, lanes 2 and 3) by 4.5 and 5.3 fold, respectively, relative to actin levels. Pretreatment of cells with OKB7 significantly reduced gp350 induced TNF-α stimulation. Infectious EBV decreased basal TNF-α transcription to almost one half (Table 2, lane 5), while heat-denatured EBV stimulated TNF-α mRNA production by 6.2 fold (lane 6). These results demonstrate conclusively that (a) U937 cells are able to respond to gp350, (b) OKB7 mAb can significantly inhibit this response, and (c) infectious and non-infectious EBV have different effects on TNF-α activation in these cells.

Secretion of TNF-α from gp350 treated cells.

The amounts of TNF-α secreted into the culture supernatants of AMM and U937 were determined by ELISA 36 hours after treatment with gp350. This time point (36 hours) was found to be the optimal for TNF-α determinations in pilot experiments. The results are depicted in Fig. 2. The gp350 treatment increased TNF-α levels in the culture supernatants as compared to the basal levels in both AMM and U937 cells (Fig. 2, compare lane 1 with 3). Prior treatment of cells with OKB7 reduced these levels more in AMM than in U937 cells. Finally, infectious EBV decreased TNF-α in these supernants as compared to the basal levels. Quite opposite to this, non-infectious EBV (lane 6) behaved almost like gp350 in increasing TNF-α levels in the culture supernatants of the treated cells. Pre-incubation of gp350-treated AMM with OKB7 or a control isotype-matched mouse monoclonal antibody showed that only OKB7 pre-treatment, but not the control antibody, significantly reduced gp350-mediated increase in TNF-α secretion (data not shown).

Analysis of the TNF-α mRNA stability and run-on transcription.

Experiments were conducted to determine whether gp350-induced TNF-α production was due to increased stability of TNF-α mRNA. To examine this, cells were treated with PMA, gp350, HSV-1 or HHV-6 for 6 hr, followed by the addition of actinomycin D (Act. D). HSV-1 and HHV-6 were used as positive controls since our previous data showed their strong stimulatory abilities on TNF-α transcription (Gosselin *et al.*, 1991, 1992a, 1992b). EBV was not included in these experiments since our previous and present results demonstrate that EBV infection down-regulates TNF-α secretion and mRNA levels in PMA-treated monocytic cells (Gosselin *et al.*, 1991, 1992a, 1992b). RNA was extracted at 6 hr post-treatment, then at 2, 4, 6, 8 and 10 hr following the addition of Act. D; RT-PCR was used to determine the relative levels of TNF-α mRNA remaining within the cells. The results demonstrate that with the PMA treatment, 50% of TNF-α mRNA degradation occurs at about 5 hr post Act. D addition (Fig. 3, top panel). Interestingly for both HHV-6 and HSV-1, the half-life ($t_{1/2}$) of TNF-α transcripts was ~5 hr; however, after 5 hr, TNF-α mRNA degraded more rapidly in HSV-1 treated cells than in HHV-6 treated cells. The most interesting result was obtained with the addition of gp350. With this glycoprotein, $t_{1/2}$ of TNF-α mRNA was extended to ~10 hr (Fig. 3, top panel) indicating that TNF-α mRNA is more stable following gp350 addition (Fig. 3, lower panel).

In order to examine the transcriptional activation of TNF-α by EBV-gp350, we performed run-on transcription experiments using AMM cell nuclei as described in the Materials and Methods. The results of a typical experiment are shown in Fig. 4. Addition of gp350 resulted in an approximate 2-fold induction at 1 and 3 hr, and a 20% decrease at 6 hr compared to basal TNF-α levels. Addition of EBV to cells resulted in no stimulation at 1 hr and no TNF-α transcripts at 3, 6 and 12 hr. These results, along with those of Fig. 3, demonstrate that EBV-gp350 not only induces TNF-α transcripts but also results in an extended TNF-α mRNA ½ life.

Activation of TNF-α gene transcription via gp350 is dependent on protein kinase C.

To continue our analysis of EBV-gp350-mediated gene activation, specific signal transduction pathway inhibitors were employed. Previous data have shown that activation of IL-6 in

primary B-lymphocytes by gp350 involves the activation of protein kinase C and protein tyrosine kinases (Tanner et al., 1996). We examined the effects of these inhibitors on the gp350-induced stimulation of TNF-α in AMM (results are shown in Table 3). The inhibitors for PKC, PI3-K and tyrosine kinases abrogated gp350-mediated increase in TNF-α mRNA. The adenyl cyclase inhibitor MDL 12330A-HCl also reduced the gp350-mediated increase in this mRNA species; this decrease, however, was not consistently seen in repeated experiments (data not shown). Thus, these experiments suggested the involvement of PKC, PI3-K and tyrosine kinases in the pg350-induced activation of TNF-α gene activation.

NF-κB binding is activated by gp350 interaction with monocytic cells.

To examine the role of transcription factors in the activation of gp350-mediated activation of TNF-α, we analyzed the binding of NF-κB proteins to the NF-κB-binding sites present upstream TNF-α coding sequences. The binding of specific transcription factors to this upstream site has been shown to mediate activation by LPS, Sendai virus and TPA (Goldfeld *et al.*, 1989, 1990). Following this previous evidence that Sendai virus is able to induce NF-κB binding to specific promoter sequences in the TNF-α gene in monocyte cells, we decided to investigate the ability of EBV-gp350 to mediate a similar effect. Therefore, we performed EMSA as described in the Materials and Methods.

Our results demonstrate that while basal levels of NF-κB binding are present in AMM, treatment of these cells with gp350, or UV-EBV produce elevated levels of binding (Fig. 5A, compare lane 1 with lanes 2 and 4). As expected, addition of infectious EBV, did not augment NF-κB binding (lane 3). To demonstrate the involvement of CR2 in this activation of NF-κB by gp350, we pretreated EBV-gp350 with either 2L10 or 72A1 mAbs (lanes 5 and 6). The blockage of gp350 binding to CR2 by 72A1 eliminated NF-κB binding (lane 6) whereas pre-incubation of gp350 with 2L10 did not have similar effect (lane 5). The specificity of the NF-κB interaction with the TNF-α-κB oligonucleotide was also confirmed through competition using unlabelled mutant and wild type oligonucleotides. While competition with mutant TNF-α-κB oligonucleotides did not greatly diminish binding (lane 8), addition of wild type TNF-α-κB did result in elimination of this complex (lane 7).

To confirm the authenticity of NF-κB binding to the TNF-α oligonucleotide, we performed supershift experiments using anti-p50 and anti-p65 antibodies. Anti-cFos antibodies were used as control. Both anti-p50 and anti-p65 specifically supershifted the complex bound to the TNF-α oligonucleotide while the control mAb did not (Fig. 5B, compare lanes 3 and 4 with lane 2). In addition to these experiments, we also examined the binding of CREB to the CRE site present upstream the TNF-α coding sequences and found that stimulation with gp350 did not induce CREB binding to this site. This binding was induced by ionomycin treatment (data not shown). Taken together, these results show that EBV-gp350 selectively stimulates NF-κB binding to upstream TNF-α sequences.

DISCUSSION

This study is a continuation of our previous reports demonstrating that EBV is capable of directly modulating the cytokine gene expression in target cells (Gosselin *et al.*, 1991, 1992a, 1992b). The earlier studies had indicated that live EBV stimulated IL-6 but downregulated TNF-α production from peripheral blood mononuclear cells and U937 monocytic cells. The results presented here show that, in sharp contrast to infectious EBV, which suppresses TNF-α production, gp350 induces the secretion of TNF-α from adherent monocyte/macrophage (AMM) cultures and from U937 cells. Interestingly, the latter effect seems due to both the increased rate of TNF-α transcription and increased stability of TNF-α mRNA.

Using immunofluorescent techniques with OKB7 and flow cytometry, we earlier demonstrated that U937 cells express the EBV receptor CR2 (Gosselin *et al.*, 1991). We had further demonstrated the binding of FITC-conjugated EBV to U937 cells while K562 cells (which lack CR2 expression) did no bind FITC-EBV (Gosselin *et al.*, 1991). Inada *et al.* (1983) demonstrated that fresh human peripheral blood monocytes did not bind C_3d (a CR2-specific ligand) but after a few hours incubation in a medium containing fetal bovine serum, they readily bound this ligand which strongly suggests the expression of CR2 on these cells. Some workers, e.g. Thieblemont *et al.* (1995) and Tedder *et al.* (1983) did not find CR2 on human monocytes and the monocytic cell line U937. However, these workers did not use OKB7 antibody which is known to react positively with CR2 (Gosselin *et al.*, 1991). The present results indicate that gp350-mediated activation of TNF-α occurs via CR2 as it could be significantly inhibited with OKB7 or EBV-neutralizing 72A1 mAb but not with isotype-matched control antibodies (i.e.: non-specific). It is conceivable that other cell types, e.g. T cells and follicular dendritic cells, expressing CR2, may also be affected by the immune modulating properties of EBV and gp350. In addition to these results, Revoltella *et al.* (1989) found that monocytic cell lines derived from children with defects in

hematopoietic development were capable of infection by EBV suggesting that along their life span, monocytic cells may be able to support EBV infection. EBV may thus modulate the function of different immunocompetent cells by deregulating their ability to synthesize cytokines, the key components of the immunoregulatory network.

Gp350/220 is the EBV envelope protein which binds specifically to the viral receptor CR2 (CD21) on target cells and initiates the infection process. It is not only the most abundant viral protein in the plasma membrane of productively infected cells but is also the most abundant protein present on the surface of EBV particles (Kieff, 1996). Being in such abundance, it is quite possible that this protein may be released in circulation or in localized tissue areas when virus infected cells are killed or virions are lysed by a variety of mechanisms. This may occur particularly in acute primary infections or in immunocompromised individuals in whom reactivations of EBV infection may occur. Another possibility in which human monocytic cells may be exposed to gp350 is by immunizing humans with this protein-based vaccines (Kieff, 1996). Under these conditions, gp350 may induce the secretion of TNF-α and immune deregulation. Thus, our present results may have implications for certain *in vivo* situations.

TNF-α is a pluripotent cytokine secreted by a number of cells. Initially described as an agent capable of stimulating cachexia/wasting, its activities have now been shown to modulate a number of cellular functions (Beutler & Cerami, 1989; Vilcek & Lee, 1991). One of the major characteristics of TNF-α is its immunomodulatory activity. Using a closely related Herpes virus model, IL-1α, IL-1β, IL-6, IL-10, IL-12, GM-CSF, and TNF-α were all augmented in HSV-1 stimulated antigen presenting dendritic cells (Ghanekar *et al.*, 1996). Our present studies demonstrate that gp350 can upregulate TNF-α production from human monocytes and monocytic cells. Interestingly, non-infectious virus shows effects similar to gp350, whereas infectious EBV, in fact, down-regulates this cytokine in these cells. The reasons for these contrasting effects of gp350 and EBV on TNF-α synthesis are

not clear. In this regard, however, it is noteworthy that the EBV gene BCRF1 encodes for viral IL-10 (vIL-10), a molecule that shares 84% sequence homology with the human IL-10 (hIL-10) protein (reviewed in Moore *et al.*, 1993). It has been shown that the expression of vIL-10 occurs within hours of EBV infection; it is therefore possible that the down-regulation of TNF-α gene expression by infectious EBV is due to the effects of vIL-10 (either alone or in association with other viral gene products). On the other hand, with respect to gp350-mediated activation of TNF-α, gp350 shed during the viremic phase of EBV infection or present on defective virions may affect adjacent cells thus causing a state of immune dysregulation characterized by the increased production of TNF-α. In this context, our results demonstrate that EBV-gp350, through the simple interaction with its receptor, is able to upregulate TNF-α synthesis.

The EBV receptor (CR2) is a member of the B cell antigen receptor complex. It occurs singly as well as in association with CD19, TAPA-1 (target for anti-proliferation-antibody-1) and CD35 (reviewed in Fearon & Carter, 1995). CR2 has a short cytoplasmic tail of 34 amino acids, which argues against its ability to transduce signals intracellularly. CR2 uses CD19 and/or TAPA-1 for transducing signals via phospholipase C and phosphatidyl inositol-3 kinase (Bradbury *et al.*, 1992). Activation of B cells causes phosphorylation of CR2 and cross-linking of CR2 by extracellular ligands (including EBV on B cells) increases cell proliferation. It has been demonstrated that C3d or a 16 amino acid peptide corresponding to the CR2 binding domain of C3d activates p53, the Ca^{+} binding protein p68, ribonucleoprotein p120 and the phosphorylation of pp105 (Barel *et al.*, 1995; Bouillie *et al.*, 1995). A report by these investigators has further demonstrated that a 34 amino acid sequence within the cytoplasmic portion of CR2 may be responsible for transmitting proliferative signals to B-lymphocytes (Frade *et al.*, 1985). Blockage of this sequence by a synthetic peptide completely inhibited EBV or C3d induced B lymphocyte proliferation through CR2 (Balbo *et al.*, 1995). In addition to the above-mentioned reports,

activated CR2 may also be involved in B cell regulation independently of CD19 or TAPA-1. Indeed, activated CR2 alone triggered tyrosine phosphorylation of intracellular components (Bouillie *et al.*, 1995) and activated PI-3-kinase through a CD19-independent pathway in B cells (Bouillie *et al.*, 1999). Our AMM cultures are devoid of CD19 suggesting a similar route of stimulation for TNF-α by gp350. Although CR2 and its associated cell surface receptors have been well characterized on B-lymphocytes, the role played by CR2 on the surface of other cells, including T-lymphocytes and monocytes, has yet to be determined.

The present studies demonstrate that gp350 induces activation of TNF-α gene involving PKC, PI3-K and tyrosine kinases and by inducing activation of NF-κB family of transcription factors but not of CREB or AP-1. Recently, it was shown that gp350 induces IL-6 production in human B cells via activation of PKC and tyrosine kinases (Tanner *et al.*, 1996). Thus, regarding activation of these enzymes, gp350 has similar effects on human B cells and monocytic cells. Sugano *et al.* (1997) have shown that activation of NF-κB is needed for a successful EBV infection and gp350 induces this activation in human B cells by binding to CR2. Thus, the role of gp350 is not just to land the virus onto its CR2-bearing target cells but also to make the inner milieu of these cells (which are resting B cells and have little or no constitutively activated NF-κB) suitable for viral replication. Our results suggest gp350 can also mediate activation of these transcription factors in human monocytic cells. However, the infectious EBV was not able to induce activation of NF-κB in these cells and these differential effects of EBV and gp350 were similar to those discussed above for TNF-α activation. The reasons for these differential effects have been discussed above.

Immune modulatory properties of the receptor-binding viral glycoproteins as described here for gp350 are not restricted to EBV. For example, the receptor-binding viral glycoprotein of HIV-1, gp120, has been well documented to modulate cytokine gene

expression upon binding to its receptor CD4 molecule both in HIV-infected and non-infected monocyte-macrophages, T-lymphocytes and glial cells (Ameglio *et al.*, 1994; Clouse *et al.*, 1991; Fantuzzi *et al.*, 1996; Koka *et al.*, 1995; Merrill *et al.*, 1989, Rieckmann *et al.*, 1991). Interestingly, gp120 also inhibits the production of IL-12 from human monocytic cells by upregulating the secretion of IL-10 from them (Taoufik *et al.*, 1997). Whether, gp350 similarly inhibits the production of IL-12 from human monocytic cells is under investigation. Furthermore, in addition to the receptor-binding proteins, viruses encode several other proteins during their life cycles that interact with various cellular factors of the host to evade the immune response (reviewed in Spriggs, 1996). With regards to gp350, its immune modulating properties may have important implications for anti-EBV vaccination. Gp350-based vaccines have proven effective in protecting animal models from EBV-induced lymphomas (Morgan *et al.*, 1988). By inducing the secretion of pro-inflammatory cytokines, the glycoprotein itself may be acting as an adjuvant.

MATERIALS AND METHODS

Cell culture and treatments.

Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors by centrifugation of heparinized whole blood over a Ficoll-Hypaque density gradient (Pharmacia). PBMC were plated on tissue culture dishes pre-treated overnight with heat denatured fetal bovine serum (FBS) and were allowed to adhere during an overnight incubation at 37° C. RPMI 1640 medium (GIBCO BRL/Life Technologies, Burlington, Ont.) containing 10 % FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 mM Hepes (pH 7.2), and 1 µg/ml gentamycin. Adherent monocyte-macrophages (AMM) were obtained by gentle scraping with a rubber policeman. These cell preparations always contained less than 3% B cells as contaminants when tested by flowcytometry using a CD19-specific monoclonal antibody (data not shown). Cells were washed twice in RPMI (0% FBS) before induction protocols were commenced. U937 cells were maintained at 37°C, 5.0 % CO_2, in RPMI 1640 medium (GIBCO BRL) supplemented with 10.0 % FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 1 µg/ml gentamycin.

For the induction of TNF-α secretion, AMM or U937 cells were treated with PMA (phorbol-myristate-acetate: 25 ng/ml, Sigma, St. Louis), EBV (strain B95-8 at a titer of $2x10^5$ EBNA-inducing units/ml), actinomycin D (Act. D, 1µg/ml, Boehringer Mannheim, Laval, Que.), or gp350 (concentration used is indicated in individual experiments).

EBV and gp350 preparation.

EBV was prepared as previously described (Menezes *et al.*, 1976). Briefly, the transforming strain, B95-8 of EBV (Miller & Lipman, 1973) was obtained from cell free supernatants of B95-8 cells. Supernatants from week old cultures of these cells were filtered through 0.45 µm filters (Nalge Labware, Corning, New York) and centrifuged at 45,000 g for 90 min at 4° C. The viral pellets were resuspended in PBS to yield 500 X concentration of virus as compared to the culture supernatants. The virus preparation was titered by the induction of nuclear antigen (EBNA) in BJAB cells (Menezes *et al.*, 1976). The viral stocks contained 2 X 10^5 EBNA-inducing units per ml. The virus preparation was heat-inactivated (56° C for one hour). UV inactivated virus was obtained by

irradiating EBV for 60 min at 265 nm. Both UV and heat-inactivated viral preparations were non-infectious as tested by their ability to induce EBNA in BJAB cells (data not shown). Preparation of herpes simplex virus-1 (HSV-1) and human herpes virus-6 (HHV-6) have been previously described (Flamand *et al.*, 1991; Menezes & Bourkas, 1980).

A truncated version of gp350 lacking the membrane anchor was produced in mouse fibroblast cell line C127 after transfection with a bovine papilloma virus-based expression system (Madej *et al.*, 1992). The transfected cells secreted gp350 in the culture medium from where it was purified using Sephacryl S300HR and gelatin agarose (Madej *et al.*, 1992). The purified protein gave bands of expected molecular weights on SDS-PAGE on silver staining, behaved similar to the native gp350 (obtained from EBV-producing human lymphoid cells) upon isoelectric focussing and was immunologically reactive as determined by its reactivity with several gp350-specific monoclonal antibodies (Madej *et al.*, 1992).

The endotoxin contents of the viral and gp350 preparations used in this study were determined using a Limulus amoebocyte lysate-based endotoxin detection kit (ICN Immunochemicals, Montreal, Canada) and were always <25 pg/ml (data not shown).

Reagents and antibodies.

Anti-CR2 monoclonal antibody (mAb) OKB7 was obtained from Ortho Diagnostic Systems (Raritan, New Jersey); it neutralized gp350 binding to target cells at 50 pg/ml. Anti-gp350 mAb 2L10 was a gift from Dr. G. Pearson (Georgetown University, Washington, D.C.). Antibody 72A1 was a gift from Dr. J. Gosselin (Universite de Laval, Québec).

In order to determine which signaling pathways were involved in the induction of TNF-α by gp350, cells were stimulated with this glycoprotein in the presence of specific inhibitors of various enzymes. These inhibitors were tyrphostin AG1478 (an inhibitor of protein tyrosine kinases, epidermal growth factor receptor and platelet-derived growth factor receptor; 100 μM), bisindoylmaleimide (a specific inhibitor of PKC; 5 μM), staurosporine (a broad-spectrum inhibitor of protein kinases; 50 nM), Ly294002 (a specific inhibitor of PI3-K; 40 μM) and MDL 12,330A-HCl (an irreversible inhibitor of adenyl cyclase, 1 mM). All these inhibitors were purchased from

Calbiochem/InterScience (Markham, Ontario, Canada) and the manufacturer-recommended concentrations were used in this study.

RNA isolation.

RNA isolation was performed using a modified guanidium thiocyanate procedure (Chomczynski & Sacchi, 1987). Briefly, cells were collected by centrifugation (1,200 g for 10 min), rinsed in phosphate buffered saline (PBS; 75 mM NaCl, 2 mM KCl, 8 mM NaH_2PO_4), and resuspended in 1 ml of solution D (4 M guanidium thiocyanate, 25 mM Na citrate, pH 7.0, 0.5 % sarcosyl, and 100 mM beta-mercaptoethanol). The cell pellet was vortexed, placed on ice for 15 min, then centrifuged in an Eppendorf-microfuge for 20 min at 4° C at 1,400 g, after the addition of equal volume of phenol-water, one tenth volume of chloroform-isoamyl alcohol and one twentieth volume of 0.5 M Na acetate (pH 4.0). The upper aqueous phase was precipitated overnight at -20° C in sterile microfuge tubes with an equal volume of isopropanol. RNA was pelleted at 14,000 g, for 20 min at 4° C, then resuspended in solution D and an equal volume of isopropanol, followed by another overnight incubation at -20° C. Total RNA was then pelleted at 14,000 g, for 20 min at 4° C, then resuspended in RNAse free water, and treated with DNAse (RQ1 DNase, Promega) for 30 min.

Reverse transcription (RT) and polymerase chain reaction (PCR) analysis.

RT was performed on total RNA (1 µg) using 5 U of Moloney Murine Leukemia Virus Reverse Transcriptase (Mo-MuLV-RT, GIBCO-BRL), and 10 pmole of random primers. The mixture was heat-denatured for 5 min at 85° C. Total reaction volume was 20 µl in buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, and 3 mM $MgCl_2$. PCR assays were performed in a total volume of 50 µl using all the RT product, in PCR buffer containing 100 mM Tris-HCl (pH 8.4), 500 mM KCl, 15 mM $MgCl_2$, 100 µg/ml BSA, and 2 mM of each of the four deoxynucleoside triphosphates (Pharmacia), 10 pmole primer A (forward primer), 10 pmole primer B (reverse primer) and 1.0 U Taq DNA polymerase (Promega). After reverse transcription, the RT products were held at 95° C for 5 min, annealed at 55° C for 1 min, extended at 72° C for 1 min, and denatured at 95°

C for 1.5 min. Thirty cycles of amplification were used. All PCR experiments included one control tube with no reverse transcription template.

PCR amplified products were resolved in a 1.0 % TAE agarose gel. Gels were denatured, neutralized, and DNA fragments were transferred onto nylon membranes (Hybond-N Nylon Membranes, Amersham) through overnight capillary blotting and UV crosslinked at 312 nm using a UV Stratalinker 1800 (Stratagene/PDI Bioscience, Aurora, Ont.). Nylon membranes were prehybridized and hybridized in Rapid-Hyb Buffer (Amersham Life Sciences, Oakville, Ont.) for 4 hr, and overnight in a Tek-Star Jr. Hybridization Oven (Bio-Can Scientific). Membranes were screened with oligonucleotide probes used in PCR amplification. Oligonucleotides were 5' end labeled with [γ^{32}P] ATP. PCR amplified products were autoradiographed overnight at -70° C and quantified through normalization with PCR amplified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which was performed simultaneously with cytokine gene amplification. Quantification was performed using the Pharmacia LKB Bromma Laser Densitometer as previously described (D'Addario *et al.*, 1990). Sequences of the oligonucleotides used in the RT-PCR analysis were as described (Arcari *et al.*, 1984; Nedwin *et al.*, 1986): TNF-α, forward primer 5'-CCCTCAAGCTGAGGGGCAGCTCAG-3'; reverse primer 5'-GGGCAATGATCCCAAAGTAGACCTG-3', GAPDH forward primer 5'-CCATGGAGAAGGCTGGGG-3'; reverse primer 5'-CAAAGTTGTCATGGATGCC-3'. The semi-quantitative nature of our RT-PCR protocol, the precautions taken and the controls used have all been previously described (D'Addario *et al.*, 1990). All experiments were performed at least twice on two independent sets of induced and uninduced samples.

Assay for determining cytokine concentration.

Concentration of TNF-α in the cell free supernatants was determined using a commercial ELISA kit (R & D Systems, Minneapolis, Minn.) according to the manufacturers instructions. Cell free supernatants were collected at 36 hr after treatment. This time point was chosen based on preliminary evidence indicating that maximal secretion of TNF-α occurred at this time (Gosselin *et al.*, 1991, 1992a, 1992b). The lowest limit of detection of TNF-α by this kit is 15 pg/ml.

Northern blot analysis.

Total cellular RNA was isolated from untreated, TPA-, EBV-, or gp350-treated cells at 6 hr using the modified guanidium thiocyanate procedure described above. Total RNA samples (5 µg each) were electrophoresed through a 1.0 % agarose formaldehyde gel (1.0% agarose, and 1X MOPS (3-morpholino-propanesulfonic-acid)) transferred onto nylon membranes by capillary blot and crosslinked as described above for the Southern transfer of PCR products. Prehybridization and hybridization was performed as previously described (Roulston *et al.*, 1992). Probes were obtained from ATCC (Bethesda, Maryland) and were generated by liberating the coding sequences for both actin and TNF-α. Pst1 cleavage of the vector pcDV1 liberated a 1.1 kb fragment of TNF-α. The preparation of the actin probe was as described (Roulston *et al.*, 1992). The filters were washed using standard protocols and exposed as described above. Between hybridization reactions, the filters were stripped of radioactivity and re-probed.

Electrophoretic mobility shift assay (EMSA).

Whole cell extracts (WCE) were prepared from AMM cells after induction with specific agents (described in each experiment). For this purpose, cells were collected by centrifugation (1,200 rpm, 10 min), washed once in 1 volume of 1 X PBS and resuspended in 3 volumes of lysis buffer (20 mM Hepes pH 7.9, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 10% glycerol, 10 mM sodium molybdate, 0.5 mM PMSF, and 1 mM DTT) containing 1 µg/ml each pepstatin, leupeptin and aprotinin protease inhibitors (all from Boehringer Mannheim, Laval, Quebec). Cells were lysed by adding 2 M KCl to a final concentration of 0.5 M KCl, gently mixed by rotation at 4° C for 30 min, and then centrifuged at 35,000 rpm for 60 min at 4° C in a Ti60 rotor (Beckman Ultracentrifuge). Supernatants were diluted to 0.1 M KCl with lysis buffer and protein concentrations were determined using the Pierce Protein Assay.

A 5-10 µg sample of crude WCE from AMM was pre-incubated with 5 µg poly (dI:dC) in nuclear dialysis buffer (NDB; 20 mM Hepes, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM KCl, 2 mM DTT, and 5 % glycerol) for 10 min at 4° C and then 20 ng of ^{32}P-end labeled double straded

DNA oligonucleotides were added to the mixture and incubated for further 20 min at room temperature. The oligonucleotides used were 5'-GGGTTTCTCCAC-3' representing NF-κB-binding site from the TNF-α promoter (-98 to -87), the mutant TNF-α oligonucleotide 5'-GAATTCACTTC-3' and the IgG-κB mutant sequence 5'-GGGGACTTTCC-3' have been previously characterized (Goldfeld and Maniatis, 1989; Goldfeld *et al.*, 1990; Roulston *et al.*, 1992). In competition assays, 200-fold molar excess of the unlabeled wild type or mutant oligonucleotide was added during pre-incubation. For mobility supershifts, which were performed to identify the binding proteins to the oligonucleotides in the EMSA, 1 μg of the p50 or p65-specific monoclonal antibodies were added during the pre-incubation period. After incubation, samples were analyzed on a 6 % native Tris-glycine PAGE, run at 150 V for 5 hr, dried and exposed to Cronex film (DuPont).

Run-on transcription analysis:

This analysis was performed to determine whether gp350 activated the transcription of TNF-α gene in monocytic cells. The procedure used was as described by Collart *et al.* (1987). Briefly, 3-5 $x10^5$ cells were washed in cold PBS, lysed in 1 ml of the lysis buffer and centrifuged at 1000 g for 10 minutes at 4° C to collect nuclei. The nuclei were resuspended in 200 μl of the transcription buffer. After 30 minutes incubation at 26° C, RNA was extracted and used in equal amounts for RT-PCR analysis of the TNF-α transcripts. The RT-PCR products were quantified by laser densitometry as described above.

ACKNOWLEDEMENTS

We are grateful to Dr. G. Pearson for 2L10 mAb, to Dr. G. Ahronheim for comments on the manuscript and to Sabrina Van Asveld for excellent secretarial assistance.

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FIGURE LEGENDS

Figure 1: RT-PCR analysis of TNF-α gene activity after treatment of AMM cells with gp350. **A)** Adherent monocyte-macrophages ($2x10^5$ cells/ml) were treated with increasing concentrations of gp350 for 6 hours, RNA was isolated and 1 μg was subjected to RT-PCR analysis. Relative mRNA was determined according to previously established protocols (D'Addario *et al.*, 1990). **B)** Time course of TNF-α gene activation by EBV-gp350. AMM cells ($2x10^5$ cells/ml) were cultured with gp350 (100 ng/ml) for increasing amounts of time, RNA was isolated and 1 μg was analyzed for TNF-α and GAPDH transcripts by RT-PCR. Lane 1: mock-treated cells; lane 2: 2 hr; lane 3: 4 hr; lane 4: 6 hr; lane 5: 8 hr; lane 6: 10 hr; lane 7: 12 hr; lane 8 : 14 hr; lane 9: 16 hr; lane 10: 24 hr; and lane 11: non-RT control. **C)** Densitometric analysis of the time course of TNF-α gene activation by gp350. The RT-PCR products obtained at different time points (Figure 1B) were quantified by laser densitometry and are depicted in this Figure in arbitrary units. The maximum increase in the TNF-α mRNA 6 hours post-treatment is evident.

Figure 2: Production of TNF-α protein in differentially treated cells. AMM and U937 cells were cultured and treated as indicated below. TNF-α protein was measured in culture supernatants by ELISA as described in the Materials and Methods. Column 1: untreated cells; column 2: PMA-treated; column 3: gp350-treated; column 4: EBV-treated; column 5: cells preincubated with OKB7 followed by gp350 treatment; and column 6: cells treated with UV-EBV.

Figure 3: TNF-α RT-PCR analysis in actinomycin D-treated monocytic cells. Following the addition of PMA (25 ng/ml), gp350 (100 ng/ml), HHV-6 (50 TCID 50/cell) or HSV-1 (50 PFU/cell) to U937 cells ($1x10^6$ cell/ml) for 6 hours Act. D was added and RNA was isolated and analyzed for TNF-α mRNA by RT-PCR. Top panel shows relative mRNA of TNF-α at different time points after Act. D additions whereas the lower panel shows the original radiographs. Lane 1: 6 hr maximal stimulation; lane 2: 2 hr post Act. D treatment; lane 3: 4 hr post Act. D treatment; lane 4: 6 hr post Act. D treatment; lane 5: 8 hr post Act. D treatment; and lane 6: 10 hr post Act. D treatment.

Figure 4: Run-on transcription of TNF-α mRNA in gp350-treated cells. Nuclei from $5x10^5$ gp350-treated and untreated AMM were isolated at different time points indicated on the x- axis of the figure and run-on transcripts for TNF-α were determined as described in Materials and Methods. Each bar in the Fig. depicts fold increase in run-on transcripts compared to the untreated cells at zero hour. Increased transcription of TNF-α mRNA in gp350-treated cells until 6 hours post-treatment is evident.

Figure 5: Activation of NF-κB in gp350-treated monocyte-macrophages. **A)** AMM cells were treated as indicated in each lane below, and whole cell extracts were prepared 5 hours later. EMSA was performed using 5 μg of protein and the oligonucleotides described in the materials and methods. Lane 1: untreated cells; lane 2: gp350-treated cells; lane 3: EBV-treated cells; lane 4: UV-EBV treated cells; lane 5: gp350 preincubated with 2L10 mAb; lane 6: gp350 preincubated with 72A1 mAb; lane 7: oligonucleotide competition using unlabelled wild type TNF-κB; lane 8: oligonucleotide competition using unlabelled mutant TNF-κB; and lane 9: oligonucleotide competition using unlabelled mutant Igγ-κB. **B)** Electromobility supershifts with NF-κB-specific antibodies. Whole cell extracts were prepared from AMM cells treated with gp350. Five μg of protein was analyzed by gel shift for binding to the TNF-α NF-κB oligonucleotide using antibodies to NF-κB p50 (~1 μg) and p65 (~1 μg) and c-fos (~1 μg). Lane 1: gp350 treated cells; lane 2: gp350 treated cells and anti-c-fos mAb; lane 3: gp350 treated cells and anti-p50 mAb; and lane 4: gp350 treated cells and anti-p65 mAb.

TABLE 1

Densitometric analysis of RT-PCR products in differentially treated AMM cells.

Lane	1	2	3	4	5	6	7	8	9
Relative Change	1X	2X ⇩	3.7X ⇧	3.1X ⇧	3.2X ⇧	4.7X ⇧	4.2X ⇧	3.2X ⇧	10X ⇩

TNF-α RT-PCR products were quantitated by laser densitometry and normalized to GAPDH levels as described in Materials and Methods. The relative increase/decrease of TNF PCR product for each cell treatment compared to the basal level was calculated and is shown above. AMM cells ($2x10^5$ cells/ml) were treated as indicated in each lane, RNA was isolated and TNF-α and GAPDH transcripts were analyzed by RT-PCR. Lane 1: mock-treated cells; lane 2: EBV-treated (B95.8, 2.0 x 10^5 EBNA units/cell); lane 3: PMA (25 ng/ml)-treated; lane 4: gp350 (100 ng/ml) treated; lane 5: both gp350 and PMA; lane 6: UV-irradiated EBV; lane 7: heat-inactivated EBV; lane 8: cells preincubated with heat-denatured OKB7 followed by gp350 stimulation, and lane 9: cells preincubated with OKB7 followed by stimulation with gp350.

TABLE 2

Densitometric analysis of the TNF-α mRNA expression in differentially treated U937 cells.

Lane	1	2	3	4	5	6
Relative Change	1X	4.5X ⇧	5.3X ⇧	1.3X ⇧	0.4X ⇩	6.3X ⇧

The levels of TNF-α RNA obtained following northern blot analysis were quantified by laser densitometry and normalized to actin levels as described in Materials and Methods. The relative increase/decrease of TNF for each cell treatment was calculated and is shown as compared to the basal TNF transcript levels. U937 monocytic cells ($1x10^6$ cells/ml) were treated with different agents, RNA was isolated and (5 μg) was analyzed by northern blot for TNF-α and actin. Lane 1: untreated cells; lane 2: PMA-treated; lane 3: gp350-treated; lane 4: cells preincubated with OKB7 followed by gp350 stimulation; lane 5: cells treated with infectious EBV, and lane 6: cells treated with UV-EBV.

TABLE 3

The effect of various inhibitors of signal transduction on gp350-induced activation of TNF-α

Lane	1	2	3	4	5	6	7
Relative Change (%)	1.0	40.0 ⇧	21.0 ⇧	31.5 ⇩	7.4 ⇧	5.7 ⇧	9.2 ⇧

The levels of TNF-α RT-PCR products were determined by laser densitometry and normalized to GAPDH levels as described in Materials and Methods. The relative increase/decrease of TNF PCR product for each cell treatment was calculated and is shown as a comparison to the basal TNF transcript levels. AMM cells ($2x10^5$ cells/ml), stimulated with gp350 (100 ng/ml) were incubated in the presence of different signal transduction pathway inhibitors. Total RNA was isolated and 1 μg was subjected to RT-PCR analysis for TNF-α and GAPDH. Concentrations for each inhibitor used are described in the Materials and Methods. Lane 1: mock-treated cells; lane 2: gp350-treated cells; lane 3: MDL 12,330A-HCl; lane 4: bisindoylmaleimide; lane 5: LY294002; lane 6: tyrphostin, and lane 7: staurosporine.

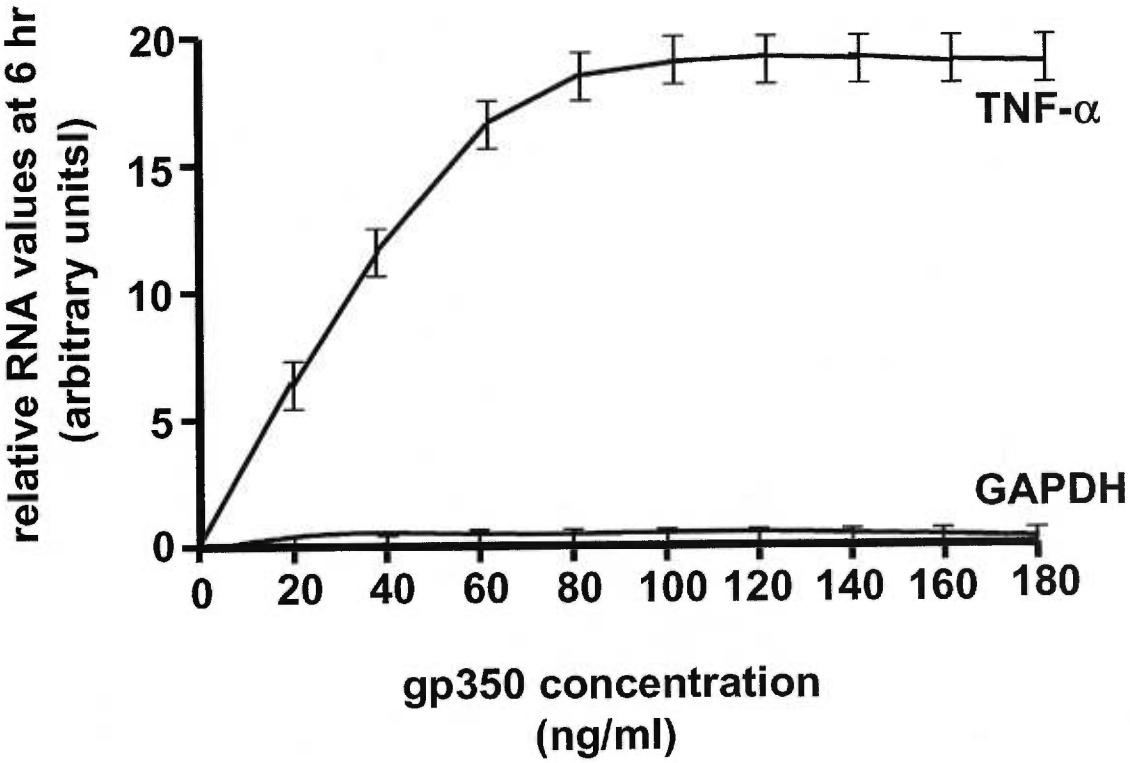
relative RNA values at 6 hr
(arbitrary units)
20
15
10
5
0
TNF-α
GAPDH
0
20
40
60
80
100
120
140
160
180
gp350 concentration
(ng/ml)

Figure 1A

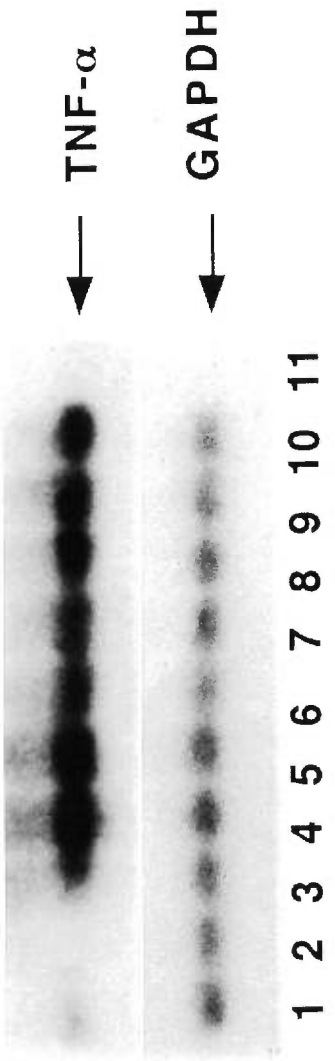

Figure 1B

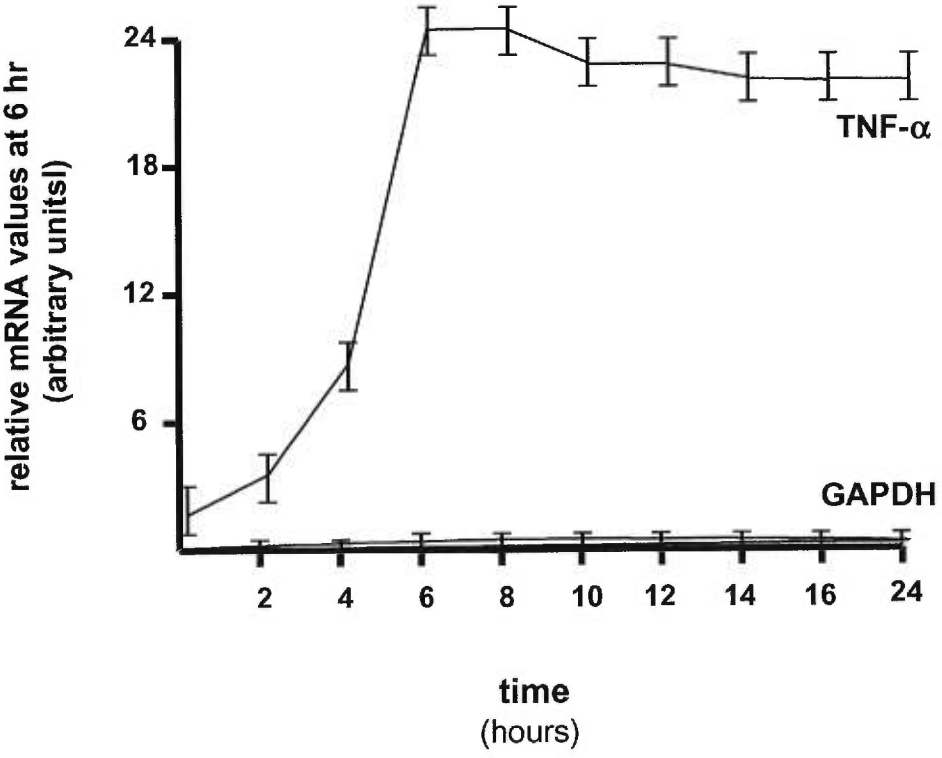
relative mRNA values at 6 hr
(arbitrary units)
24
18
12
6
TNF-α
GAPDH
2
4
6
8
10
12
14
16
24
time
(hours)

Figure 1C

Figure 2

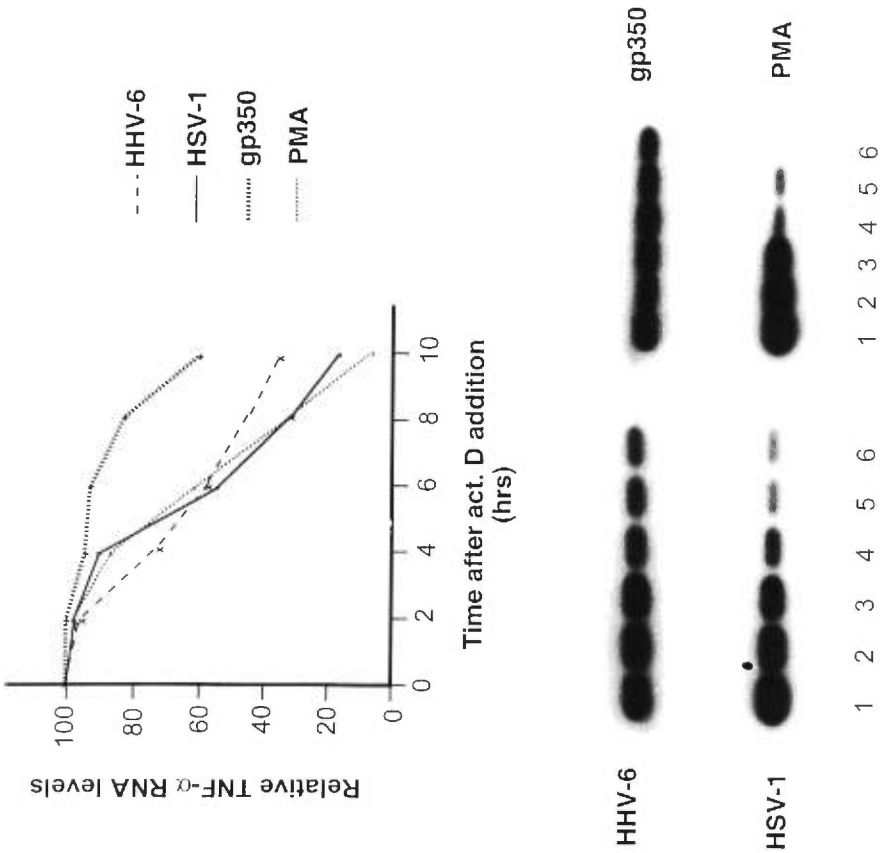
HHV-6
HSV-1
gp350
PMA
Relative TNF-α RNA levels
100
80
60
40
20
0
0
2
4
6
8
10
Time after act. D addition
(hrs)
HHV-6
gp350
HSV-1
PMA
1 2 3 4 5 6
1 2 3 4 5 6

Figure 3

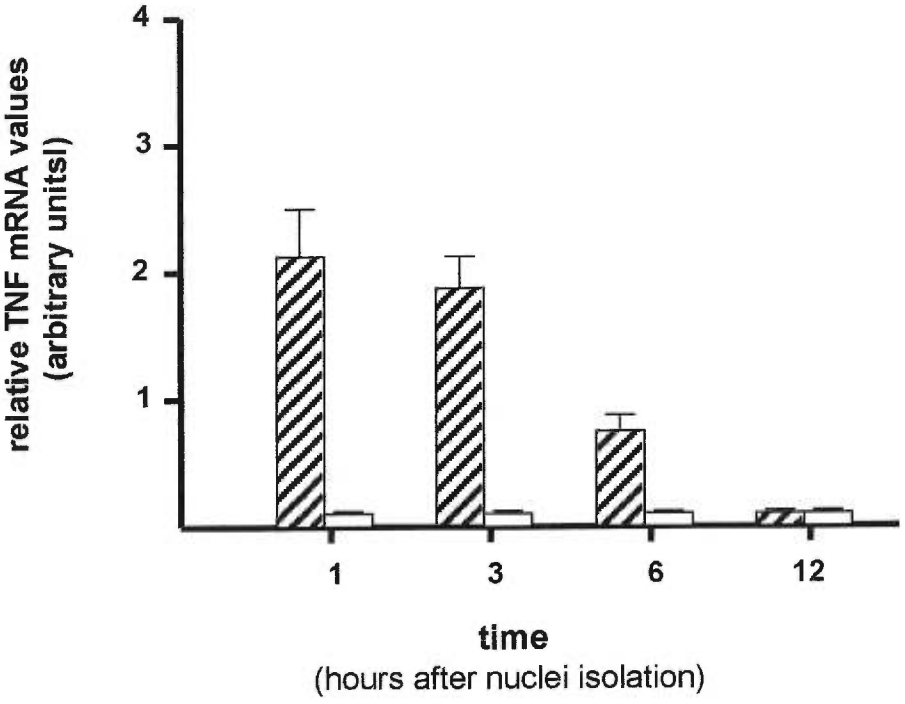
relative TNF mRNA values
(arbitrary unitsl)
4
3
2
1
1
3
6
12
time
(hours after nuclei isolation)

Figure 4

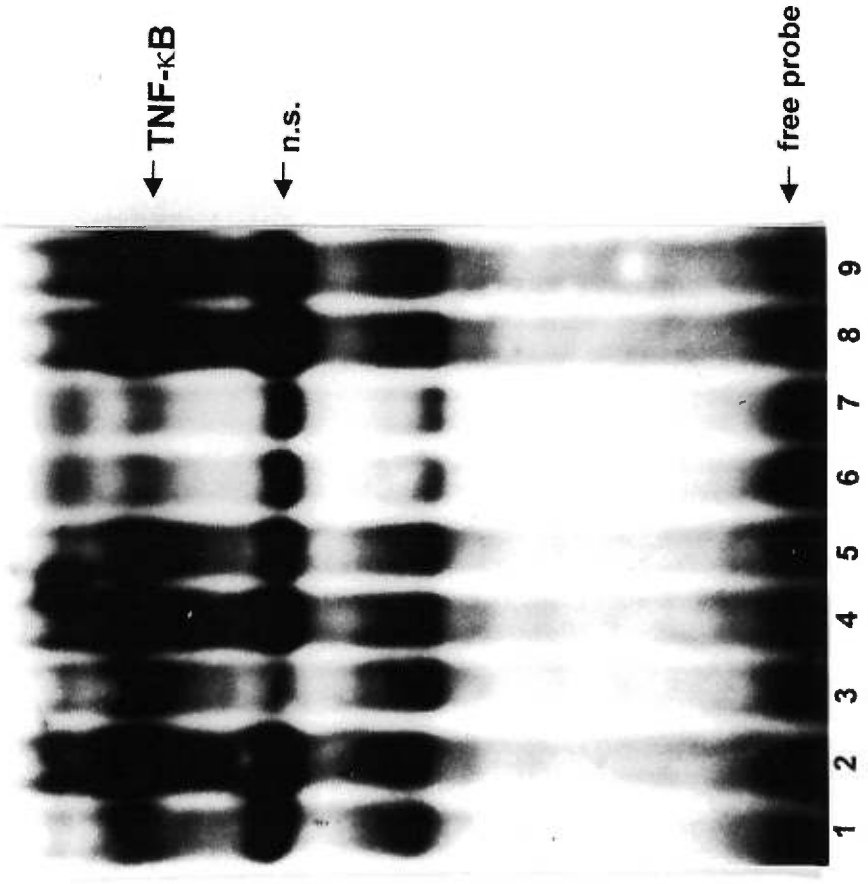

Figure 5A

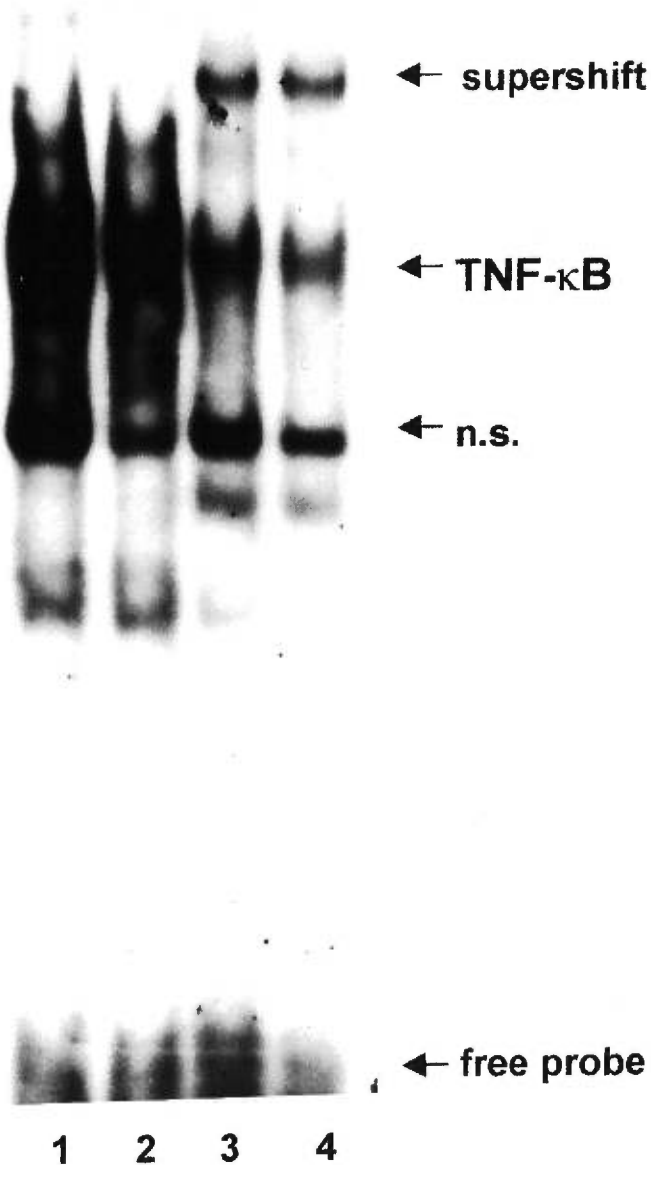

Figure 5B

Epstein-Barr virus and its glycoprotein-350 upregulate IL-6 in human B-lymphocytes via CD21, involving activation of NF-κB and different signalling pathways.

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Running title: IL-6 activation by EBV-gp350 via CD21 and NF–κB.

Key words: EBV-gp350, interleukin-6, signal transduction, transcription factors.

This study was supported by a grant from the Medical Research Council of Canada.

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Abstract

Epstein-Barr virus (EBV) is a ubiquitous and highly immunotropic gamma herpesvirus that infects more than 90% of humans worldwide. Its pathogenicity leads to a number of diseases including tumors that result from EBV's ability to immortalize certain target cells. EBV utilizes CD21/CR2 as its receptor to initiate the infectious process. EBV binds to CR2 through its major envelope glycoprotein-350 (gp350) and is also a remarkable immunomodulating agent. We had previously shown that EBV is capable of modulating the synthesis of a number of cytokines. We now show that while both purified recombinant gp350 (rgp350) and EBV upregulate IL-6 mRNA synthesis, EBV-induced IL-6 gene activation occurs for a significantly longer period of time (ie: 12 hr for EBV as compared to 6 hr for rgp350). Moreover, the half-life of EBV-induced IL-6 mRNA was also significantly longer (10 hr) than that of mRNA induced by rgp350 (4 hr). Both EBV and gp350 enhance the binding of the NF–κB transcription factor, as determined by band-shift and augment NF-κB mediated activation of a CAT reporter plasmid. Furthermore, we demonstrate that while the activation of IL-6 gene expression by gp350 is mediated primarily by the protein kinase C pathway, EBV does not appear to mediate its effects through one particular signal transduction pathway. To our knowledge this is the first report showing that the binding of a herpesvirus envelope glycoprotein to CR2 results in the upregulation of IL-6 gene expression through the activation of the NF-κB transcription factor.

Introduction

Epstein-Barr virus is a lymphotropic human herpesvirus which is associated with several diseases including infectious mononucleosis, African Burkitt's lymphoma, nasopharyngeal carcinoma, oral hairy leukoplakia, Hodgkin's and non-Hodgkin's lymphomas and B-cell lymphomas in immunocompromised individuals [1]. Primary infections usually occur in early childhood presumably following transmission of the virus via saliva and are generally self-limiting and symptomless. In the developed industrialized countries, these infections are often delayed until adolescence and cause mononucleosis. By adulthood more than 90% of humans become infected and life-long carriers of this virus. It is now well established that CD21 (type II complement receptor or CR2) serves as a receptor for EBV. The major EBV envelope glycoprotein 350/220 (hereafter referred to as gp350) binds to this receptor and initiates the infectious process. The receptor has been demonstrated on a wide variety of human cells including B- and T-lymphocytes, thymocytes, epithelial cells, follicular dendritic cells, endothelial cells, gastric carcinoma cells, neutrophils and monocytes [2-11]. In vitro infection of B-lymphocytes results in cellular immortalization, blast cell formation, cell adhesion, surface CD23 expression and increased RNA synthesis [1]. EBV-induced B-lymphocyte immortalization and proliferation are facilitated by autocrine and paracrine growth factors that are only now beginning to be understood.

Interleukin-6 (IL-6) is a pleiotropic cytokine that regulates many hematopoietic cell functions. Originally described as interferon-β2, this cytokine belongs to a family of related proteins [IL-6, IL-11, oncostatin M, leukemia inhibitory factor and ciliary-neurotrophic factor; 12]. IL-6 is a critical cytokine in inducing the host response to stress and injury through its ability to stimulate the acute phase proteins of the liver. IL-6 stimulates growth and differentiation of murine progenitor cells and synergizes with other growth factors to modulate the activities of T cells, hepatocytes and hematopoietic progenitor cells [12,13]. IL-6 may also play a critical role during EBV infection; this cytokine is thought to act in a paracrine and autocrine manner to facilitate growth of EBV-

immortalized B cells [14]. In vitro, EBV-immortalized cells secrete IL-6, express the IL-6 receptor and use this cytokine as a growth factor [14]. The exact role and mechanism used by IL-6 in these cells has never been elucidated as the IL-6 receptor is capable of activating a number of intracellular signal transduction pathways [13]. IL-6 treatment of murine myeloid cells was shown to activate the nitric oxide synthase gene promoter through direct stimulation of unique transcription factors POU and octamer binding protein-1 [15]. Sugano et al. [16] found that EBV-genome positive immortalized B-cells re-treated with EBV responded by activating NF-κB leading to viral reactivation. These authors found that CD21-mediated reactivation of EBV by NF-κB occurred through the Wp viral gene promoter. While these data demonstrate NF-κB dependent EBV activation, no studies have ever examined the activation of cellular genes through CD21 in primary B-lymphocytes.

We have previously shown that herpesvirus-treated monocytic cells respond by modulating the transcription and translation of pro-inflammatory cytokines IL-1, IL-6 and TNF [17-19]. Our present data show that EBV- or gp350-treated B-lymphocytes respond through CD21/CR2 to activate IL-6 gene expression specifically via activation of NF-κB involving different signaling pathways.

Materials and Methods

Lymphocyte preparation and culture.

Human B-lymphocytes were purified from peripheral blood of healthy donors using a Ficoll-Hypaque density gradient and subsequent treatment with magnetic beads coated with anti-CD19 monoclonal antibodies according to the manufacturers instructions (Immunotech, Coulter, Burlington, Ont.). This purification resulted in ≥ 98% pure B-lymphocyte preparation as determined by CD19 expression. Cells were maintained for a maximum of 7 days in Dulbecco's modified Eagle medium (GIBCO BRL/ Life Technologies, Burlington, Ont.) containing 10 % (v/v) fetal bovine serum (FBS) 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 mM Hepes (pH 7.2), and 1 µg/ml gentamycin.

EBV and recombinant gp350 (rgp350) preparation.

EBV was prepared as previously described [3]. Briefly, the transforming strain B95-8 of EBV [20] was obtained from cell-free supernatants of B95-8 cultures. Supernatants from week-old cultures of these cells were filtered through 0.45 µm filters (Nalge Labware, Corning, New York) and centrifuged at 45,000 g for 90 min at 4 °C. The viral pellets were resuspended in PBS to yield 500 X concentration of virus as compared to the culture supernatants. The virus preparation was titered by the induction of nuclear antigen (EBNA) in BJAB cells [3]. After preliminary experiments for optimal IL-6 induction by EBV, we chose to use the viral preparation at a concentration of 2 X 10^5 EBNA-inducing units per ml throughout this study, unless otherwise specified for a particular experiment.

The rgp350 preparation used for these experiments was a gift from Dr. A. Morgan (University of Bristol, U.K.). This rgp350 was produced using a bovine-papilloma virus expression system and purified using Sephacryl-S300HR and gelatin agarose [21]. In preliminary experiments, we tested this rgp350 for its ability to bind to CD21/CR2; it bound specifically to the surface of CR2-positive

Raji cells. This was detected by immunofluorescence and flow cytometry using gp350-specific 2L10 monoclonal antibody. The purity of this rgp350 preparation was verified by silver staining through a 6% SDS-PAGE. Preliminary experiments had indicated that 100 ng/ml of rgp350 produced optimal results (data not shown). Unless otherwise specified in the text, this concentration was then used throughout this study. The endotoxin contents of EBV and rgp350 preparations used in this study were determined using a Limulus amoebocyte lysate-based endotoxin detection kit (ICN Immunochemicals, Montreal, Canada) and were always < 20 pg/ml.

Antibodies and other reagents.

Rgp350-specific mAb 2L10, which does not block rgp350 binding to CR2, was a gift from Dr. G. Pearson (Georgetown University, Washington, D.C.), and anti-rgp350 mAb 72A1, which blocks binding of rgp350 to CR2, was kindly provided by Dr. J. Gosselin (Université Laval, Quebec, Canada). Specific monoclonal antibodies for p50, p65, NF-IL-6 and c-Fos were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); each of these was used in electrophoretic mobility shift assays (EMSA) at 1 μg per reaction. In certain experiments, control treatments included TPA at 25 ng/ml (Sigma) and actinomycin D (Act. D; 1 μg/ml; Boerhinger Mannheim).

In order to determine which signaling pathways were involved in the induction of IL-6 by rgp350, the cells were stimulated with this glycoprotein in the presence of specific inhibitors of signal transduction pathways. The inhibitors used were: tyrphostin AG1478 (an inhibitor of protein tyrosine kinases, epidermal growth factor receptor and platelet-derived growth factor receptor; 100 μM); protein kinase G inhibitor (5 μM); herbimycin (a specific inhibitor of protein tyrosine kinase; 7 μM); bisindoylmaleimide (a specific inhibitor of PKC; 5 μM); LY294002 (a specific inhibitor of PI-3K; 40 μM); staurosporine (a broad spectrum inhibitor of protein kinases; 50 nm) and MDL 33A-HCl (an irreversible inhibitor of adenyl cyclase; 1 mM). All these inhibitors were purchased

from Calbiochem/InterScience (Markham, Ontario) and the concentrations for each inhibitor were those recommended by the manufacturer and are those shown in parentheses in this paragraph.

Assay for determining cytokine concentration.

The concentrations of IL-6 in the culture supernatants were determined using a commercial ELISA Kit (R&D Systems, Minneapolis, Minn.) according to the manufacturer's instructions. The lowest limit of detection of IL-6 by this kit was 3.1 pg/ml. Each assay was performed in duplicate using three independent sets of purified B-lymphocytes.

RNA isolation.

RNA isolation was performed using a modified guanidium thiocyanate procedure [22]. Cells were collected by centrifugation (1,200 g for 10 min), washed with phosphate buffered saline (PBS; 75 mM NaCl, 2 mM KCl, 8 mM NaH_2PO_4) and resuspended in 1 ml of solution D (4 M guanidium thiocyanate, 25 mM Na citrate, pH 7.0, 0.5 % sarcosyl, and 100 mM β-mercaptoethanol). The cells were vortexed, placed on ice for 15 min, then centrifuged in an Eppendorf-microfuge for 20 min at 4° C at 14,000 g, after adding an equal volume of phenol/water, one tenth volume of chloroform-isoamyl alcohol and one twentieth volume of 0.5 M Na acetate (pH 4.0).

Reverse transcription (RT) and polymerase chain reaction (PCR) analysis.

RT was performed on total RNA (1 μg) using 5 U of Moloney Murine Leukemia Virus Reverse Transcriptase (Mo-MuLV-RT, GIBCO-BRL) and 10 pmoles of random primers. The mixture was heat-denatured for 5 min at 85° C. Total reaction volume was 20 μl in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT and 3 mM $MgCl_2$. PCR assays were performed in a total volume of 50 μl using all the RT product in a PCR buffer containing 100 mM Tris-HCl (pH 8.4), 500 mM KCl, 15 mM $MgCl_2$, 100 μg/ml BSA and 2 μM of each of the four deoxynucleoside

triphosphates (Pharmacia), 10 pmole primer A (forward primer), 10 pmole primer B (reverse primer) and 1.0 U Taq DNA polymerase (Promega). The PCR reactions involved an initial incubation at 95° C for 5 min and then annealing at 55° C for 1 min, extension at 72° C for 1 min, and denaturation at 95° C for 1.5 min. Thirty cycles of amplification were used. All PCR experiments included one control tube with no reverse transcription step and were performed twice on two independent sets of treated cells. PCR amplified products were resolved in a 1.0% TBE agarose gel, transferred to nylon membranes and validated by probing with ^{32}P-end labeled oligonucleotide probes that were used for PCR as described [23]. Each assay was performed in duplicate in three independent sets of cultured B-lymphocytes.

Quantification of the PCR products was performed using the Image-Quant phosphorimager (Molecular Dynamics Technologies, Sunnyvale, CA) and normalized with a PCR-amplified housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequences of the oligonucleotides used in the RT-PCR analysis were as described [24]: IL-6 forward primer 5'-CCAAGAATCTAGATGCAATAAA-3'; IL-6 reverse primer 5'-GCCCATTAACAACAACAATCTG-3'; GAPDH forward primer 5'-CCATGGAGAAGGCTGGGG-3'; reverse primer 5'-CAAAGTTGTCATGGAGCC-3'. The semi-quantitative nature of our RT-PCR protocol, the precautions taken and the controls used were as described previously [23].

Run-on transcription analysis:

A time course analysis was performed to determine whether gp350 or EBV activate the transcription of the IL-6 gene. The procedure used was as described by Collart et al. [25] except that our nuclei were isolated at various time following EBV or gp350 treatment. Briefly, 3-5 x 10^5 cells were washed in cold PBS, lysed in 1 ml of the lysis buffer and centrifuged at 1000 g for 10 minutes at 4 °C to collect nuclei. The nuclei were resuspended in 200 µl of the transcription buffer [25]. After 30

minutes of incubation at 26 °C, RNA was extracted and used in equal amounts for RT-PCR analysis of the IL-6 transcripts. The RT-PCR products were quantified as described above.

Electrophoretic mobility shift assays (EMSA):

In order to determine the NF-κB binding activity in the rgp350-treated and control cells, EMSA was performed. For this purpose whole cell extracts were prepared as described [26]. Briefly, cells were pelleted by centrifugation 4 hr after the start of the cultures, washed with ice-cold PBS, and resuspended in 0.5 ml of the lysis buffer (20 mM Hepes, pH 7.9, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 10% glycerol, 10 mM sodium molybdate, 1 mM DTT) containing protease inhibitors i.e. 0.5 mM PMSF, and 1 μg/ml of each of the following inhibitors: pepstatin, leupeptin and aprotinin (all of these inhibitors were obtained from Boehringer Mannheim, Laval, Quebec). Cells were lysed by adding 2 M KCl drop wise to a final concentration of 0.5 M KCl, gently mixed by rotation at 4° C for 30 min and then centrifuged at 45,000 g for one hour at 4° C in a Beckman Ultracentrifuge. The supernatants were diluted to 0.1 M KCl with the lysis buffer and protein concentrations of the supernatants were determined using Bio-Rad protein assay.

For EMSA, double-stranded DNA oligonucleotides representing the NF-κB site in the IL-6 promoter were used. A double-stranded DNA oligonucleotide corresponding to the c-AMP response element (CRE) was also used in some assays and is 5'-CAGATGAGCTCAT-3". In addition, the following oligonucleotides were used: IL-6 NF-κB wild type 5'-GTGGGATTTTCCCAT-3', IL-6κB mutant 5'-GTGGGATTTTAGACT, IL-6 AP-1 wild type 5'-GTGCTGAGTCACTAA-3', NF-IL-6 wild type 5'-TCATTGCACAATCTT-3', and NF-IL6 mutant 5'-TCAGATATCAATCTT-3'. All these oligonucleotides have been described [27-29]. Ten μg of the protein from whole cell extracts were preincubated with 5 μg of poly (dI:dC) for 10 min at 4° C and then 20 ng of the ^{32}P-end labeled oligonucleotides were added to the mixture and incubated for 20 min at room temperature. In competition assays, 200-fold molar excess of the unlabelled

oligonucleotides was added to the mixture during pre-incubation. For mobility supershifts, monoclonal antibodies (1 μg) specific to p50, p65 or NF-IL6 specific were added during the pre-incubation period. After incubation, the samples were analyzed on a 6% native Tris-glycine PAGE, migrated at 150 V for 5 hours, dried and exposed to X-ray films for different lengths of time.

Transfection of CAT plasmids.

Human CD19-positive B-cells were isolated from whole blood as described above and transfected by electroporation using the Bio-Rad Gene Pulser (Bio-Rad, Mississauga, Ont.) at 200 V and 500 μF. Briefly, cells (2 x 10^6 cells/ml) were mixed on ice with 10 μg of plasmid DNA in 0.5 ml PBS, pulsed once, returned to ice for 10 min and then plated in RPMI 1640 with 10 % FBS. Specific IL6CAT vectors used have been described [27,28]. Suspensions were incubated for an additional 36 hours prior to induction with specific agents. Twenty-four hours after treatment with specific inducers, cells were washed with ice-cold PBS and lysed. Whole-cell proteins were isolated through three successive 2-minute periods of freezing and thawing. Lysates were centrifuged and membrane fractions were discarded. For individual CAT assays, 50-100 μg of whole cell protein was assayed for 2 hrs at 37 °C and analyzed by ELISA for CAT protein according to the manufacturer's instructions (Boerhinger Mannheim).

Results

EBV-rgp350 and EBV upregulate IL-6 transcription.

B-cells treated with EBV-rgp350 or EBV responded by strongly enhancing IL-6 gene transcription. Time course experiments using a constant amount of rgp350 (100 ng/ml) or EBV (2 x 10^5 EBNA inducing units per ml) resulted in an approximate 9 fold and 5 fold induction of IL-6 mRNA over basal levels, respectively (Figure 1A and 1B). A low basal level of constitutive IL-6 RNA was present that was quickly stimulated by both EBV and rgp350 to peak at approximately 8 hr following treatment; the GAPDH housekeeping gene was not affected by either treatment. Interestingly, treatment with infectious EBV resulted in a sustained elevated production of IL-6 mRNA that was evident even after 48 hr of EBV treatment (Fig. 1B, lane 12). This sustained activation of IL-6 gene expression was not evident following 12 hr of rgp350 treatment (Fig. 1A, lane 10) indicating that infectious EBV sustains IL-6 gene activation for longer periods of time.

We then analyzed the transcriptional activation and half-life of IL-6 mRNA. The results, shown in Figure 2A and 2B, demonstrate that while both EBV and rgp350 activated IL-6 transcription, EBV was a stronger inducer. EBV treatment resulted in an RNA molecule with a half-life of ~10 hr while rgp350 treatment produced a half-life of ~ 4 hr (Fig. 2A). In addition to this difference, EBV also induced IL-6 more strongly at 6-12 hr following nuclei isolation (Fig. 2B, lane 6). Analysis of rgp350-mediated IL-6 gene activation showed a negligible amount at 6 hr and an undetectable amount of IL-6 mRNA at 12 hr post-treatment (Fig. 2B, lane 6). Together, these results demonstrate that while both EBV and rgp350 are able to enhance IL-6 gene transcription, EBV does so to greater levels and for longer period of time.

EBV and rgp350 induce IL-6 protein synthesis via interaction with CD21/CR2.

We first determined the induction of IL-6 protein by EBV and rgp350 preparations in purified B-cell cultures and then confirmed the specificity of this IL-6 activation. In response to rgp350, IL-6

protein was induced by ~3 fold, i.e. in relation to its basal levels of 180 ±25 pg/ml: to 480 ±45 pg/ml for rgp350 and 520 ±60 pg/ml for EBV (Figure 3, compare columns 2 and 3 with column 1). The specificity of CD21 involvement was determined by the use of mAb 2L10, an antibody that reacts with rgp350 but does not inhibit its binding to CD21/CR2. In this case, pre-incubation did not inhibit the induction of IL-6 protein following treatment with either EBV-rgp350 or infectious EBV, i.e. IL-6 protein was measured at 410 ±35 pg/ml and 440 ±20 pg/ml, respectively (Fig. 3, columns 5 and 6). In comparison, pretreatment of rgp350 or EBV with mAb 72A1, an antibody that blocks rgp350 interaction with CR2, strongly suppressed induction of IL-6 protein as compared to untreated preparation. Figure 3, columns 8 and 9 demonstrate the 80% decrease in IL-6 production following pre-incubation of rgp350 or EBV with mAb 72A1, respectively.

Control experiments using TPA and mAbs alone were included and demonstrate the specificity of both the EBV and rgp350-CD21 interaction (Fig. 3, columns 4, 7, 10, 11 and 12). Other control experiments using isotype specific mAbs IgG1, IgG2a, IgG2b, 72A1 and 2L10 alone on cells demonstrated that the interaction of EBV or rgp350 with CD21/CR2 is responsible for the observed induction of IL-6 RNA and protein from B-lymphocytes (data not shown). These results demonstrated that the activation of IL-6 by rgp350 or EBV is specific and reproducible at both the RNA and protein levels and is not due to the ancillary effects of the mAbs themselves e.g. through their Fc receptors.

EBV and rgp350 induce binding of NF-κB and NF-IL6 proteins to the IL-6 promoter.

To determine if rgp350 or EBV mediate their effects through different transcription factors, we performed a series of EMSA analyses examining transcription factors previously known to activate the IL-6 gene. In order to determine the concentration of EBV or the rgp350 necessary for maximal induction of NF-κB, both virus and glycoprotein were titrated and EMSA was performed (Figure 4). Both EBV and rgp350 specifically stimulated the binding of NF-κB proteins to an IL-6κB specific

oligonucleotide at concentrations that were essentially similar to those necessary for IL-6 gene activation (~2 x 10^5 EBNA inducing units/ml for EBV and ~100 pg/ml of rgp350) (Fig. 4, lanes 5, 6 and 11, 12). Basal levels of NF-κB binding were observed without viral or glycoprotein stimulation, but these were augmented ~6 to ~8 fold by EBV and rgp350, respectively (Fig. 4, compare lane 1 with 5-6 and lane 7 with 11-12). Analysis of NF-IL6 yielded similar results while studies using oligonucleotides specific for AP-1 (activator protein-1) and CREB (cyclic-AMP responsive element binding protein) binding sites did not produce any appreciable induction (data not shown).

To examine the specificity of EBV-induced NF-κB activation, a series of competition EMSA experiments were performed. Low basal levels of NF-κB binding were present in untreated extracts (Figure 5A, lane 1) and these levels were induced 6-8 fold by EBV-gp350 or live EBV (lanes 2 and 3). Specific rgp350- or EBV-CD21 interaction was demonstrated using mAb 72A1 that eliminated greater than 80% of binding using rgp350 or EBV (Fig. 5A, compare lanes 5 and 6 with lanes 2 and 3, respectively). Pre-incubation with mAb 2L10, which does not inhibit interaction of rgp350 with CD21, partially reduced NF-κB binding (i.e. by 15%) induced by EBV or rgp350 (Fig. 5A, lanes 8 and 9). Control experiments using TPA as a positive control were also performed (Fig. 5A, lanes 4, 7 and 10). In addition, controls using mAbs 72A1 or 2L10 alone demonstrate that they do not significantly affect NF-κB binding on their own (Fig. 5A, lanes 11 and 12); similar controls using the mouse isotype mAbs IgG1, IgG2a and IgG2b did not demonstrate any enhanced NF-κB binding (data not shown).

To confirm the presence of authentic NF-κB proteins in the EMSA analysis, competition and supershift experiments were performed. Addition of a 200-fold molar excess of unlabelled wild-type NF-κB completely eliminated binding, while addition of a mutant NF-κB competitor did not (Fig. 5B, compare lanes 2 and 3 with lane 1). The elimination of the non-specific complex (n.s.) with competition (lane 2) may indicate that these complexes are NF-κB-related or are associated with NF-κB proteins. To confirm the authenticity of NF-κB subunit proteins p50 and p65, mAb to p50

and p65 specifically supershifted the NF-κB complex and eliminated rgp350 induced NF-κB binding (Fig. 5B, lanes 4 and 5, respectively). The specificity was further confirmed through the addition of ancillary antibodies to c-fos and CREB, both of which did not produce a supershifted complex (Fig. 5B, lanes 6 and 7).

We then extended the analysis to examine NF-IL6, a transcription factor known to modulate the transcriptional activation of the IL-6 gene. Basal NF-IL6 binding was observed and was augmented by the addition of rgp350 or EBV (Fig. 6A, compare lane 1 with lanes 2 and 3). Pretreatment of either rgp350 or EBV with mAb 72A1 resulted in a specific decrease in NF-IL6 binding (Fig. 6A, compare lanes 5 and 6 with lanes 2 and 3), while pre-incubation with mAb 2L10 did not decrease NF-IL6 binding (Fig. 6A, lanes 8 and 9). Lanes 11 and 12 again demonstrate that neither mAb, alone, affects basal NF-IL6 binding (Fig. 6A, lanes 4, 7 and 10 are positive controls using TPA induction). To show the specificity of NF-IL6 binding, we pre-incubated EMSA extracts with anti-NF-IL6 mAb and produced a supershifted complex (Fig. 6B, lane 1) that was not present upon incubation with anti-NF-κB p50 or p65 (Fig. 6B, lanes 2 and 3). As indicated above, similar experiments were performed using the AP-1 and CREB oligonucleotide, but EBV or rgp350 induced neither (data not shown). As with the NF-κB analyses, controls were also performed using mouse isotype mAbs for 72A1 and 2L10; in both cases no stimulation of NF-IL6 was observed. These results demonstrate conclusively that EBV and rgp350 are able to activate the NF-κB and NF-IL6 proteins through the CD21/CR2 receptor and also show, through the lack of AP-1 and CREB activation, that a differential stimulation of nuclear factors occurs.

Upregulation of IL-6 by EBV and rgp350 via NF-κB and NF-IL-6 transcription factors.

The EMSA analysis showed that EBV and EBV-gp350 (rgp350) were able to induce NF-κB and NF-IL6 binding. In order to determine the importance of these transcription factors in IL-6 gene activation, transfection of wild type IL-6CAT and mutant NF-κBCAT and NF-IL6CAT plasmids

was performed. The results show that the wild type IL-6CAT produces a low basal level of acetylation that is significantly increased with the treatment of B-lymphocytes with either rgp350 or EBV (Fig. 7, compare columns 1, 2 and 3). Both rgp350 and EBV induced wild type IL-6CAT by approximately 4.8 and 5 fold, respectively. Using a plasmid containing the IL-6 promoter with a mutant NF-κB sequence, rgp350 or EBV mediated activation was significantly reduced (Fig. 7, compare columns 5 and 6 with 2 and 3, respectively). Interestingly, an IL-6 promoter construct containing a mutated NF-IL6 sequence still showed strong activation following treatment with either rgp350 or EBV (Fig. 7, compare columns 8 and 9 with columns 2 and 3, respectively). These results show that the NF-IL6 sequence may not be necessary for the CR2/CD21 mediated activation of the IL-6 gene by rpg350 or EBV. These results, along with the EMSA data demonstrate that while both NF-κB and NF-IL6 are activated to bind their respective oligonucleotides following gp350 or EBV treatment of B-lymphocytes, only NF-κB may be required for the activation of IL-6 by gp350 or EBV through CD21/CR2. These results do not exclude the involvement of other transcription factors in the activation of IL-6 through CR2/CD21 but show a difference in the effects of NF-κB as compared to those of NF-IL6.

EBV and rgp350 differentially activate specific signal transduction pathways.

In order to determine the importance of specific signal transduction pathways necessary for IL-6 gene activation by EBV or rgp350 through NF-κB or NF-IL6, we treated B-lymphocytes with specific signal transduction inhibitors. These were aimed at protein kinase A, protein kinase C, protein kinase G, adenylate cyclase, phospholipase-C, phosphotidylinositol-3-kinase, protein tyrosine kinase or calcium ion dependent protein kinase signal transduction pathways. While previous studies have examined the necessity of the above mentioned signal transduction pathways in IL-6 gene activation, no studies have ever compared the activation of IL-6 using either virions or a viral envelope glycoprotein that binds to the receptor on the target lymphocyte.

The results show that while rgp350 and EBV were both able to augment IL-6 mRNA production, effects of specific signal transduction inhibitors differed between the two activators. Thus, for rgp350, IL-6 gene transcription was dependent on the protein kinase C pathway while for EBV it was not specifically dependent on one particular pathway (Fig. 8, compare lane 4 for both the upper EBV and lower rgp350 mediated activation of IL-6). For rgp350, the reduction using the protein kinase C inhibitor was ~90% while for EBV its was reduced only ~40% (Fig. 8, lane 4 of the upper panel). Comparatively, the protein tyrosine kinase inhibitor herbimycin inhibited both the EBV- and rgp350-mediated activation of IL-6 by equal percentage (~20%). Lastly, the PI-3-K inhibitor LY294002 suppressed IL-6 induction by approximately 20% while not affecting rgp350's effects significantly (Fig. 8, lane 10). These results suggest that the EBV-mediated induction of IL-6 involves several signaling pathways that would differ from those of rgp350.

Discussion

The results presented here show for the first time that EBV and its major envelope glycoprotein gp350 enhance the production of interleukin-6 through the NF-κB transcription factor. Our results show that gp350 or EBV, through CD21/CR2, induce a time course dependent increase in the levels of IL-6 mRNA and induce a sustained level of this RNA for up to 8 hr. In addition, rgp350 treatment of B-lymphocytes stimulated the expression of the NF-κB transcription factor through a PKC-dependent signal transduction pathway.

Virus-induced immunomodulation has been described in many viral systems. Our previous work with EBV, herpes-simplex virus-1 (HSV-1) and human herpesvirus-6 (HHV-6) demonstrated differential activation of TNF-α, IL-1β and IL-6 in peripheral blood mononuclear cells [17-19]. EBV was found to activate the transcription and translation of IL-6 while decreasing the production of TNF-κB in these cells [18]. The treatment of these same cells with herpes-simplex virus type -1 (HSV-1) also resulted in the activation of IL-6 and not TNF-κ while treatment with human herpesvirus-6 (HHV-6) resulted in increase of both cytokines [19]. Carmack et al. [31] using HSV-1 and HSV-2 in a T-lymphocyte cell model have found that T-cell proliferation and the production of IL-2 and IFN-α were elevated in HSV-2 and IgG2 treated T-lymphocytes; this increase was not applicable to all cytokines, as IL-4 and IL-10 production was not significantly affected. In other studies, when HSV-1 was added to trigeminal ganglia it was found to induce a number of cytokines and chemokines including IL-2, IL-6, IL-10, IFN-α, TNF-α and RANTES; all these were not induced in untreated ganglia [32]. Our present results demonstrate that EBV, another herpesvirus is able to augment IL-6 gene expression through the interaction of its glycoprotein gp350 with the cellular receptor CD21/CR2. This increased expression of IL-6 occurs predominantly via the PKC signal transduction pathway involving activation of the NF-κB family of transcription factors.

Another herpesvirus known to affect immune activities is cytomegalovirus (CMV). CMV is a member of the beta herpesvirus family and a prevalent pathogen in immunocompromised patients.

CMV is known to directly increase IL-6 and IL-2 receptor expression in lung transplant recipients experiencing CMV pneumonitis and augment serum TNF-α in liver transplant patients with CMV disease [33-35]. More specifically, CMV, through its immediate early gene products (IE1 and IE2) increases TNF from monocytes and not fibroblasts [36] and has been shown to selectively activate the interferon immediate early response genes and the interferon regulatory factor (IRF) transcription factor complex [37].

CR2 is a 140-kDa type II integral membrane protein that belongs to the complement family of proteins [38-40]. CR2 occurs singly as well as associated with CD19, TAPA-1 (target for anti-proliferation antibody-1) and CD35; together, CR2, CD19 and TAPA-1 utilize the phospholipase-C and PI-3-K pathways as signal transduction agents [38-40]. It is known that C3d or a 16 amino acid peptide corresponding to the CR2-binding domain of C3d results in the phosphorylation of pp105 in CR2 positive cells [41-43]. CR2 interacts intracellularly with the tumor suppressor protein p53, a p68 Ca^{++} binding protein and the ribonucleoprotein p120 [42]. More recently, these authors showed independent activation of CR2 (i.e. without CD19 involvement) leading to the phosphorylation of intracellular phosphatidylinositol-3-kinase [44] and demonstrating that CR2, with its short cytoplasmic tail, is capable of transmitting signals through the cytoplasmic membrane. Our results demonstrate that beyond the direct activation of well-established signal transduction pathways, gp350 interaction with CD21/CR2 enhances the binding of transcription factors NF-κB and NF-IL-6 to the IL-6 promoter and directly activates IL-6 expression as determined by transfection assays.

The association between EBV and IL-6 is unequivocal. IL-6 is secreted into culture supernatants following EBV-induced immortalization of B-lymphocytes and induces B-cell proliferation [14]. The mechanism underlying the EBV-induced IL-6 production has never been elucidated. Previous data have demonstrated the activation of IL-6 protein following treatment of B-cells with gp350 [45], but no study has ever examined the intracellular pathways activated following the interaction of EBV or its gp350 with its CD21 receptor. Our data now suggest that specific transcription factors

may be involved in the stimulation of cellular genes by EBV and/or their proteins. Sugano et al. [16] found that EBV or rgp350 bound to CD21 specifically reactivated latent EBV in B-lymphocytes through the stimulation of NF-κB binding to the Wp promoter. Activation was immediate (within 30 min), was reproduced through binding of mAb OKB7 to CD21 and involved the PKC signal transduction pathway.

NF-κB is a family of structurally and functionally related peptides that regulate transcription of viral genes (HIV, CMV, SV40, and Adenoviruses), genes coding for cell surface receptors (MHC class I ($H\text{-}2K^b$), IL-2 receptor-α chain, T cell receptor-β, and -β_2 microglobulin), VCAM, cytokine genes (TNF-α, IFN-β, GM-CSF, G-CSF, IL-6, IL-2, IL-1β, and TNF-β) and genes encoding for several transcription factors (IRF-1, IκB-α, *c-myc*, and NF-κB p50) [30,46]. NF-κB binds to DNA as a dimer composed of either of its subunits, including RelA/p65, p50, p52, c-Rel and Rel B. NF-IL6 is a protein that was initially found to regulate the expression of IL-6 but is now known to modulate the expression of other cytokine genes including TNF-α, IL-8 and G-CSF [47]. NF-IL6 is exquisitely sensitive to LPS challenge and activates the release of IL-6 and other acute phase proteins from the liver [47]. Both regulate the expression of IL-6 in a variety of different tissues including B-lymphocytes and synovial cells [28,48]. In multiple myeloma bone marrow plasma cells, the simple act of cellular adhesion is sufficient to induce the binding of NF–κB and NF-IL-6 proteins to the IL-6 promoter [28]. In synovial cells, IL-6 acts in an autocrine manner to activate its own production as well as the binding of NF-κB and NF-IL6 proteins [48]. Our results using EMSA analysis show that while both rgp350 or EBV are able to induce NF-κB and NF-IL-6 binding to their respective oligonucleotides, only NF-κB appeared to be necessary for IL-6 gene activation.

IL-6 is a family of cytokines that effect a number of hematopoietic and immunoloigcal functions. The importance of IL-6 in EBV-induced pathology is underscored by the role of this cytokine in EBV-related diseases (described above). This cytokine, when released into serum, induces the proliferation of T-cells, plasmocytes and induces B-cell proliferation [12,13]. Following rhinovirus

treatment, for example, IL-6 mRNA and protein are elevated in nasal epithelial cells concomitant with the increased activity of NF-κB and AP-1 transcription factors [29]. Using this virus model, NF-κB subunits p50, p52 and p65 were found to increase within minutes of rhinovirus addition; abrogation of the NF-κB binding site in the IL-6 promoter resulted in significantly decreased rhinovirus-induced IL-6 promoter activity [29]. Interestingly, rhinovirus produced all the above-mentioned effects without the need for infection; i.e. interaction with the viral ICAM-1 receptor was sufficient for rhinovirus to induce these effects. Our results using EBV/gp350 with the CD21/CR2 receptor are comparable to those reported by Zhu et al. [29] with the rhinovirus; we further show that NF-κB is activated following EBV or gp350 interaction with the viral receptor CD21/CR2 and that either is sufficient to activate IL-6 through their respective promoter sequences.

In conclusion, we have shown that the interaction of EBV or its envelope glycoprotein 350 with B-cells via CD21 results in the upregulation of the interleukin-6 gene expression through the activation of NF-κB involving the stimulation of protein kinase C for gp350 and multiple signaling pathways for EBV.

Acknowledgments:

We are grateful to Drs. A. Morgan, G. Pearson, and J. Gosselin for their generous gifts of recombinant gp350, mAb 2L10 and mAb 72A1, respectively. We thank Ms. Sabrina Van Asveld for secretarial assistance.

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Figure 1 RT-PCR analysis of IL-6 mRNA in rgp350 and EBV treated B-lymphocytes. B-lymphocytes (2 x 10^5 cells/ml) were treated at different times with 100 ng/ml of rgp350, and the cells were then harvested for the determination of IL-6 mRNA and GAPDH by RT-PCR as described in Materials and Methods. **A**: Lane 1: untreated cells; lane 2: 30 min rgp350 treatment; lane 3: 60 min rgp350 treatment lane 4: 2 hr rgp350 treatment; lane 5: 3 hr rgp350 treatment; lane 6: 4 hr rgp350 treatment; lane 7: 6 rgp350 treatment; lane 8: 8 hr rgp350 treatment; lane 9: 10 hr rgp350 treatment; lane 10: 12 hr rgp350 treatment, and lane 11: non-IL-6 RT control. **B**: Lane 1: untreated cells; lane 2: 30 min EBV treatment; lane 3: 60 min EBV treatment lane 4: 2 hr EBV treatment; lane 5: 3 hr EBV treatment; lane 6: 4 hr EBV treatment; lane 7: 6 EBV treatment; lane 8: 8 hr EBV treatment; lane 9: 10 hr EBV treatment; lane 10: 12 hr EBV treatment; lane 11: 24 hr EBV treatment; and lane 12: 48 hr EBV treatment.

Figure 2A Run-on transcription analysis of IL-6 in EBV- and rgp350-treated B-lymphocytes. Following the addition of EBV or rgp350 to B-lymphocytes, nuclei were isolated, RNA was prepared and analyzed for IL-6 by RT-PCR. Top panel shows the original radiographic data while the bottom panel shows the relative RNA of IL-6 at different time points (following cell treatment). Lane 1: basal transcription; lane 2: 30 min post treatment; lane 3: 1 hr post treatment; lane 4: 3 hr post treatment; lane 5: 6 hr post treatment and lane 6: 12 hr post treatment.

Figure 2B IL-6 RT-PCR analysis in actinomycin D-treated B-lymphocytes. Following the addition of EBV or rgp350 to B-lymphocytes for 6 hr, Act. D was added and RNA was isolated and analyzed for IL-6 mRNA by RT-PCR. Top panel shows the original radiographs while the bottom panel depicts the relative mRNA of IL-6 at different time points after Act. D addition. Lane 1: 6 hr maximal stimulation; lane 2: 1 hr post Act. D treatment; lane 3: 2 hr post Act. D treatment; lane 4: 4 hr post Act. D treatment; lane 5: 8 hr post Act. D treatment and lane 6: 12 hr post Act. D treatment.

Figure 3 Production of IL-6 protein in differentially treated B-lymphocytes. B-lymphocytes were cultured in the presence of TPA, rgp350 or EBV. Thirty-six hours later, cell-free culture supernatants were collected. The concentration of IL-6 was determined using a commercial ELISA Kit. This figure shows average concentrations of IL-6 ± SE from three replicate cultures: Column 1: untreated cells; columns 2, 5 and 8: rgp350-treated; columns 3, 6 and 9: EBV-treated; columns 4, 7 and 10: TPA-treated. Columns 5, 6 and 7 were EBV- or rgp350-treated cells pretreated with mAb 2L10 while columns 8, 9 and 10 were EBV- or rgp350-treated pretreated with mAb 72A1. Column 11 is mAb 72A1 alone and column 12 is mAb 2L10 alone.

Figure 4 Binding of NF-κB proteins to the IL-6-κB oligonucleotide in rgp350- or EBV-treated B-lymphocytes. B-cells were treated with increasing amounts of EBV or rgp350 and whole cell extracts (WCE) were prepared four hours later. IL-6 NF-κB protein binding was determined by EMSA using 10 μg of the WCE and oligonucleotides containing the IL-6-κB site as described in Materials and Methods. Lane 1: unstimulated cells; lane 2: EBV-treated (0.1 x 10^5 EBNA inducing U/ml); lane 3: EBV-treated (0.5 x 10^5 EBNA inducing U/ml); lane 4: EBV-treated (1.0 x 10^5 EBNA inducing U/ml); lane 5: EBV-treated (2.0 x 10^5 EBNA inducing U/ml); lane 6: EBV-treated (5.0 x 10^5 EBNA inducing U/ml); lane 7: untreated cells; lane 8: rgp350-treated (50 pg/ml); lane 9: rgp350-treated (75 pg/ml); lane 10: rgp350-treated (100 pg/ml); lane 11: rgp350-treated (150 pg/ml) and lane 12: rgp350-treated (200 pg/ml).

Figure 5A Activation of NF-κB in differentially treated B-lymphocytes. B-cells were treated as indicated in each lane below, and whole cell extracts (WCE) were prepared 4 hours later. EMSA was performed using 10 μg of protein and the oligonucleotides described in the Materials and Methods. Lane 1: untreated cells; lane 2: rgp350-treated cells; lane 3: EBV-treated cells; lane 4: TPA-treated cells; lane 5: rgp350 preincubated with 72A1 mAb; lane 6: EBV preincubated with 72A1 mAb; lane 7: rgp350 preincubated with 2L10 mAb; lane 8: EBV preincubated with 2L10 mAb; lane 9: TPA preincubated with 2L10 mAb; lane 10: TPA preincubated with 72A1 mAb; lane 11: 2L10 mAb alone and lane 12: 72A1 mAb alone.

Figure 5B Gel super-shift with NF-κB-specific antibodies. WCE were prepared from B-lymphocytes treated with rgp350. Ten μg of protein was analyzed by EMSA for binding to the IL-6 NF-κB oligonucleotide using antibodies to NF-κB p50 (~1 μg), p65 (~1 μg) and c-fos (~1 μg). Lane 1: rgp350-treated cells; lane 2: competition using wild-type unlabeled IL-6 NF-κB oligonucleotide; lane 3: competition with mutant unlabeled IL-6 NF-κB oligonucleotide; lane 4: rgp350-treated cells and anti-p65 mAb; lane 5: rgp350-treated cells and anti-p50 mAb; lane 6: rgp350-treated cells and anti-c-fos mAb and lane 7: rgp350-treated cells and anti-CREB mAb.

Figure 6 Activation of NF-IL6 in differentially treated B-lymphocytes. B-cells were treated as indicated in each lane below, and WCE were prepared 4 hours later. EMSA was performed using 10 μg of protein and the oligonucleotide described in the Materials and Methods. **A**: Lane 1: untreated cells; lane 2: rgp350 treated cells; lane 3: EBV treated cells; lane 4: TPA-treated cells; lane 5: rgp350 preincubated with 72A1 mAb; lane 6: EBV preincubated with 72A1 mAb; lane 7: rgp350 preincubated with 2L10 mAb; lane 8: EBV preincubated with 2L10 mAb; lane 9: TPA preincubated with 2L10 mAb; lane 10: TPA preincubated with 72A1 mAb; lane 11: 2L10 mAb alone and lane 12: 72A1 mAb alone. **B:** NF-IL6 specific proteins were analyzed for binding through EMSA super-shift analysis. Lane 1: rgp350-treated cells and anti-NF-IL6 mAb; lane 2: rgp350-treated cells and anti-NF-κB p65 mAb; lane 3: rgp350-treated cells and anti-NF-κB p50 mAb and lane 4: rgp350-treated cells and c-fos mAb.

Figure 7 Upregulation of IL-6 gene expression by the NF-κB sequence as determined by CAT analysis. B-lymphocytes were transfected by electroporation and whole cell extracts were isolated and analyzed by ELISA (described in Materials and Methods). Fifty μg of total cellular protein was tested in ELISA assays for expression of CAT protein as described by the manufacturer. The bar graph shows the percentage of acetylated product for each transfection and treatment (indicated below each lane) where the open bar denotes unstimulated cells, the hatched bars denote the rgp350-treated cells, and the solid dark bars represent the EBV-treated cells. Lane 10 demonstrates a pUCAT negative control and lane 11 denotes a HIV-IIIBCAT positive control. Fold induction is also indicated and is relative to each untreated cell transfection with the same plasmid.

Figure 8 IL-6 gene transcription was analyzed in activated B-lymphocytes in the presence of inhibitors of different enzymes. B-lymphocytes were cultured in the presence of rgp350 or EBV with or without specific inhibitors of different enzymes that are involved in various signal transduction pathways. The cells were harvested six hours after the start of the cultures and processed for the determination of IL-6 and GAPDH mRNAs by RT-PCR. The lanes show mRNA from cells treated with the following; lane 1: untreated cells; lane 2: stimulated cells (rgp350 and EBV); lane 3: inducer plus tyrphostin; lane 4: inducer plus bisindoylmaleimide; lane 5: inducer plus staurosporin; lane 6: inducer plus D609; lane 7: inducer plus herbimycin; lane 8: inducer plus MDL 12,330A HCL; lane 9: inducer plus inhibitor of protein kinase G and lane 10: inducer plus LY294002. The above panel is the RT-PCR data while the lower panel shows the quantified levels as described in the Materials and Methods.

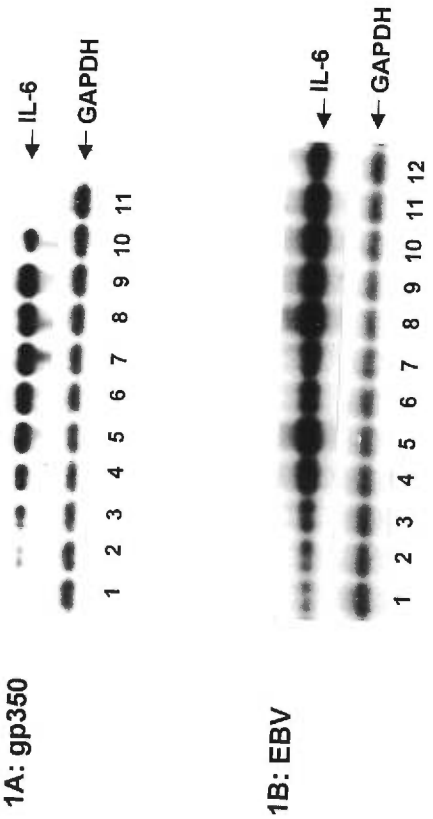
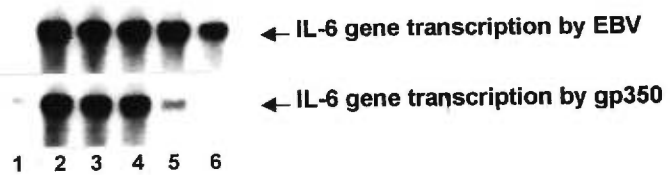
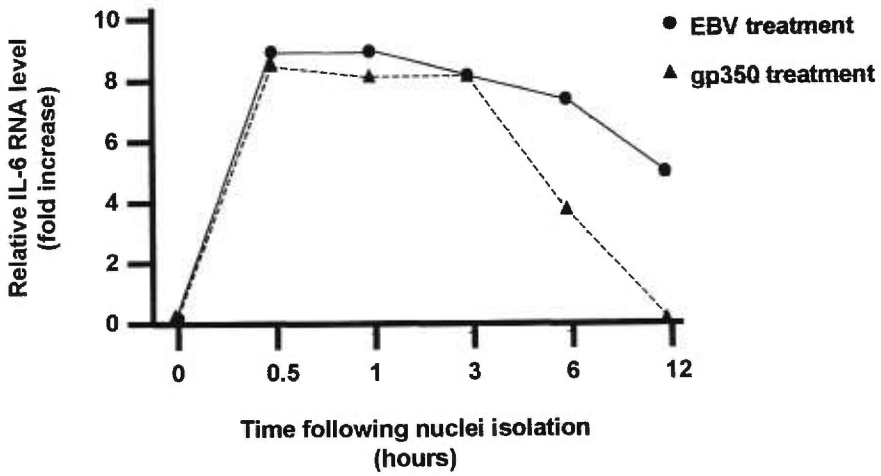

1A: gp350
IL-6
GAPDH
1 2 3 4 5 6 7 8 9 10 11
1B: EBV
IL-6
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12

Figure 1

Figure 2A


IL-6 gene transcription by EBV
IL-6 gene transcription by gp350
1 2 3 4 5 6


Relative IL-6 RNA level
(fold increase)
10
8
6
4
2
0
0
0.5
1
3
6
12
Time following nuclei isolation
(hours)
EBV treatment
gp350 treatment

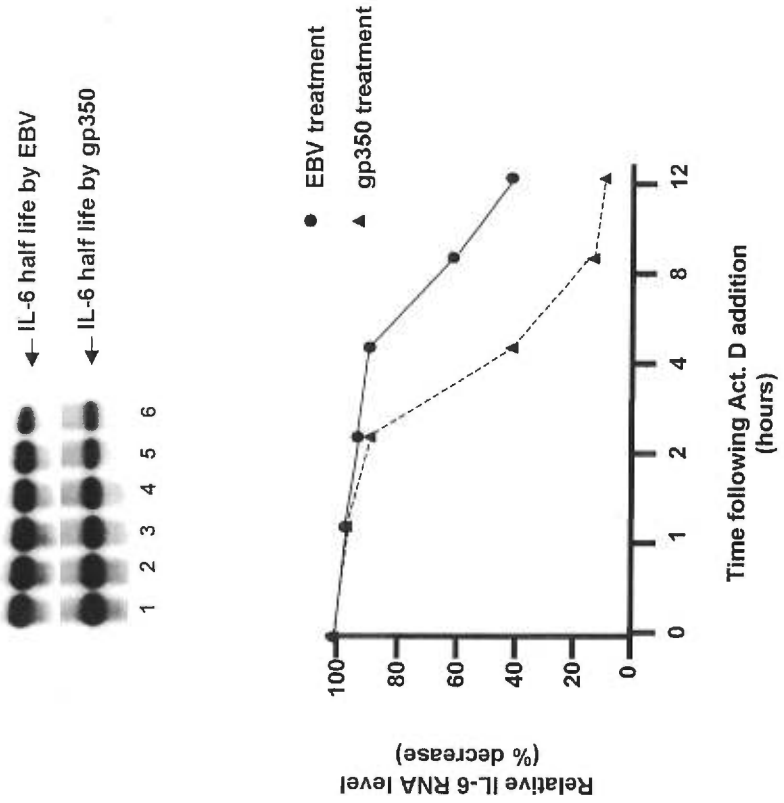

← IL-6 half life by EBV
← IL-6 half life by gp350
1 2 3 4 5 6
EBV treatment
gp350 treatment
Relative IL-6 RNA level
(% decrease)
100
80
60
40
20
0
0 1 2 4 8 12
Time following Act. D addition
(hours)

Figure 2B

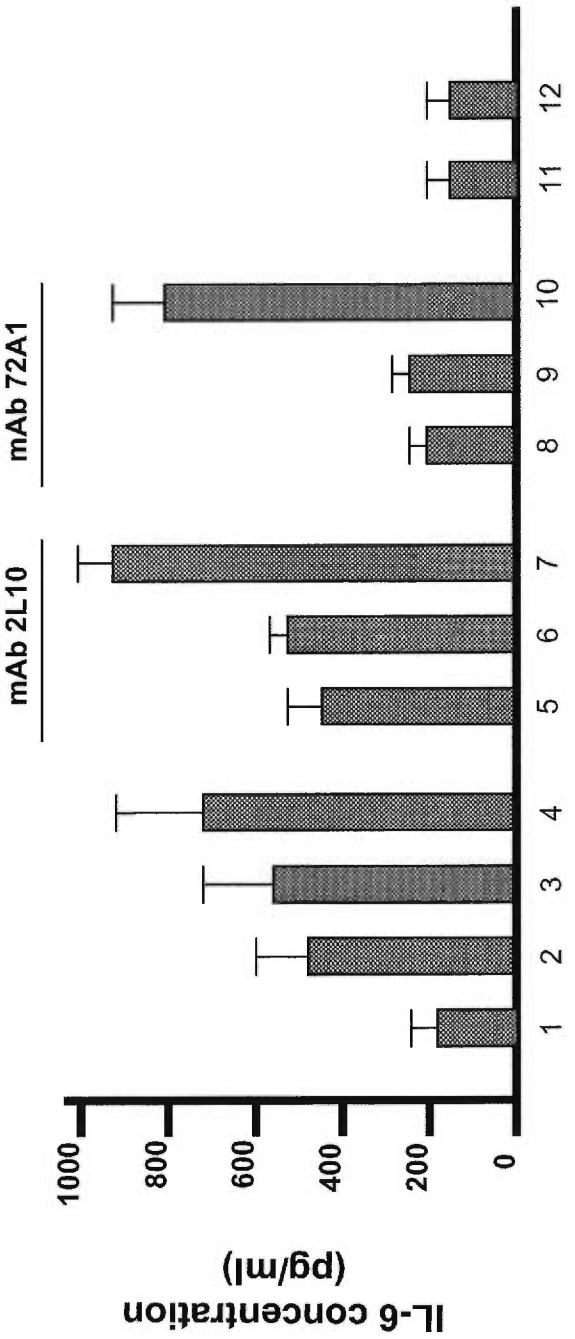

IL-6 concentration
(pg/ml)
1000
800
600
400
200
0
1
2
3
4
5
6
7
8
9
10
11
12
mAb 2L10
mAb 72A1

Figure 3

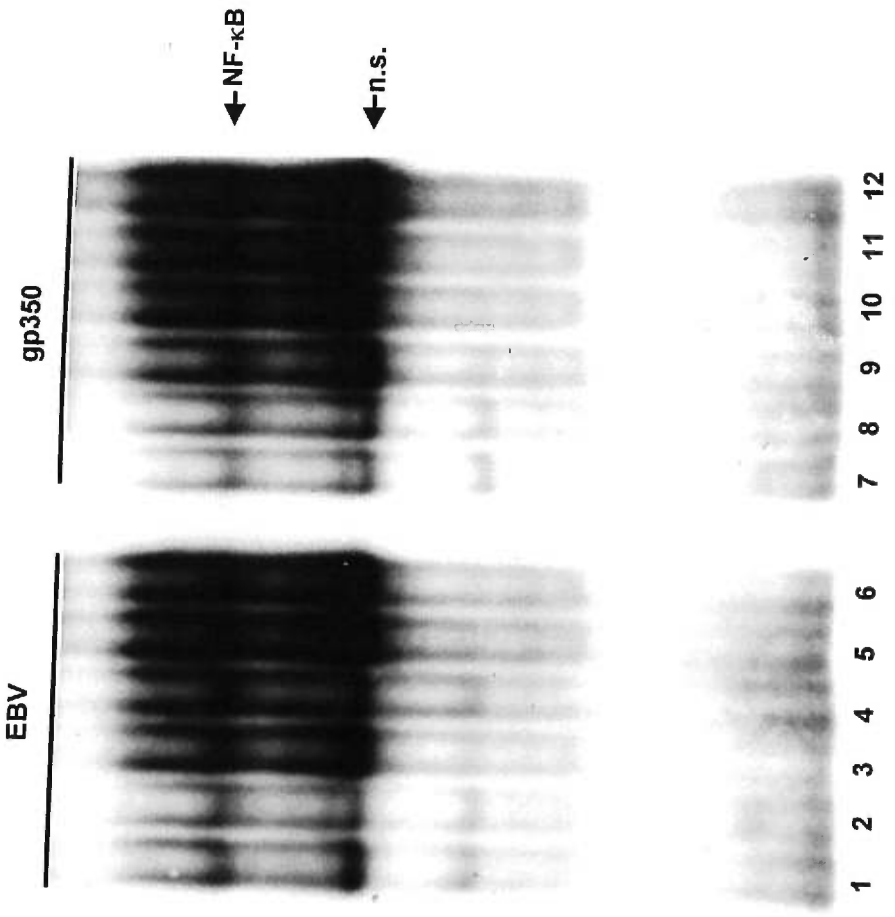


Figure 4

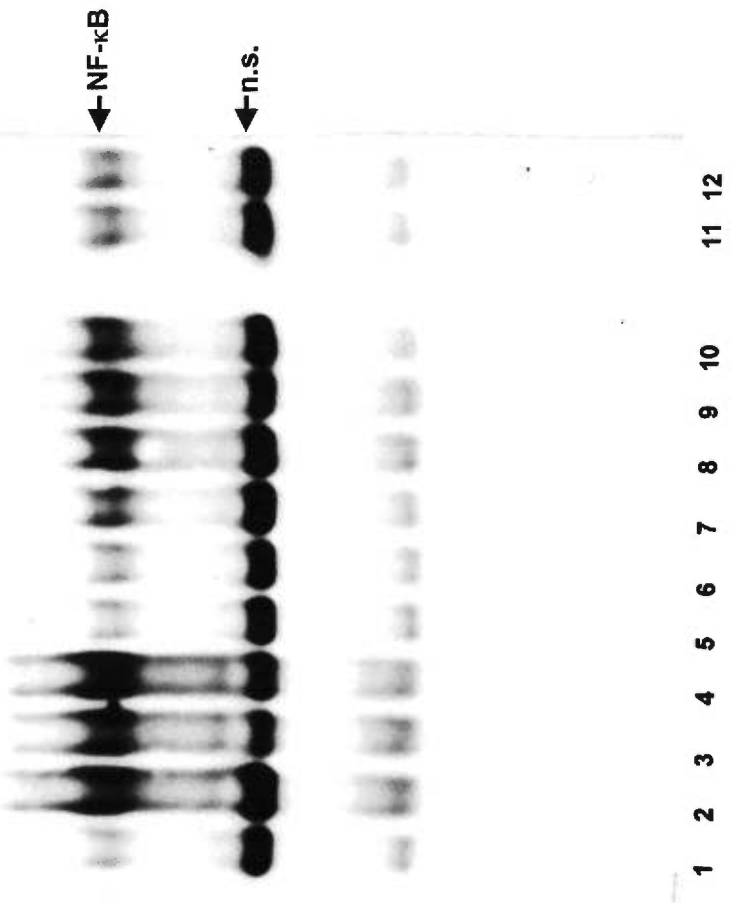


Figure 5A

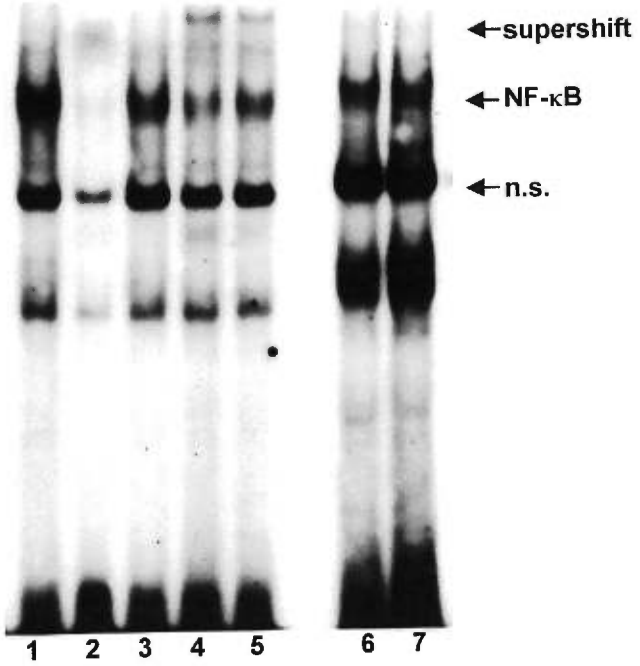


Figure 5B

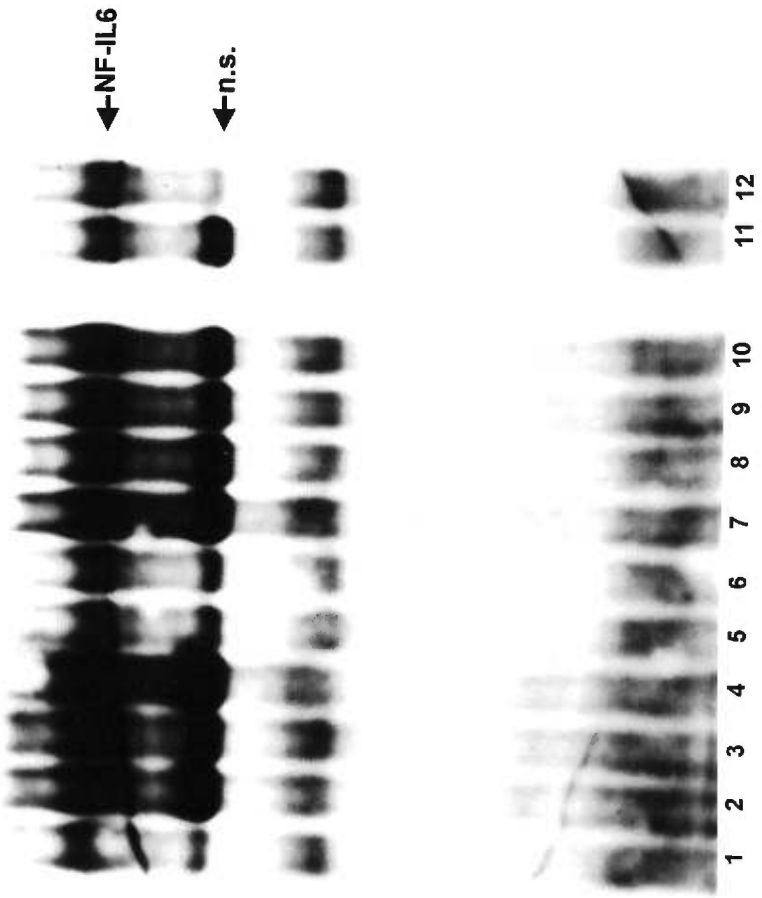


Figure 6A

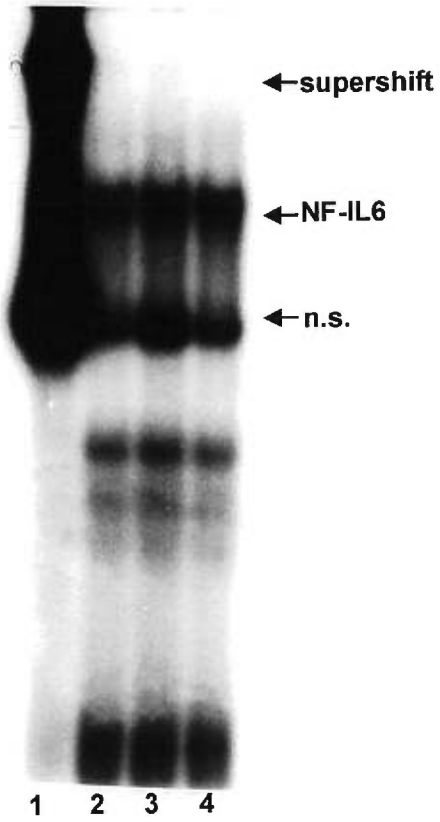


Figure 6B

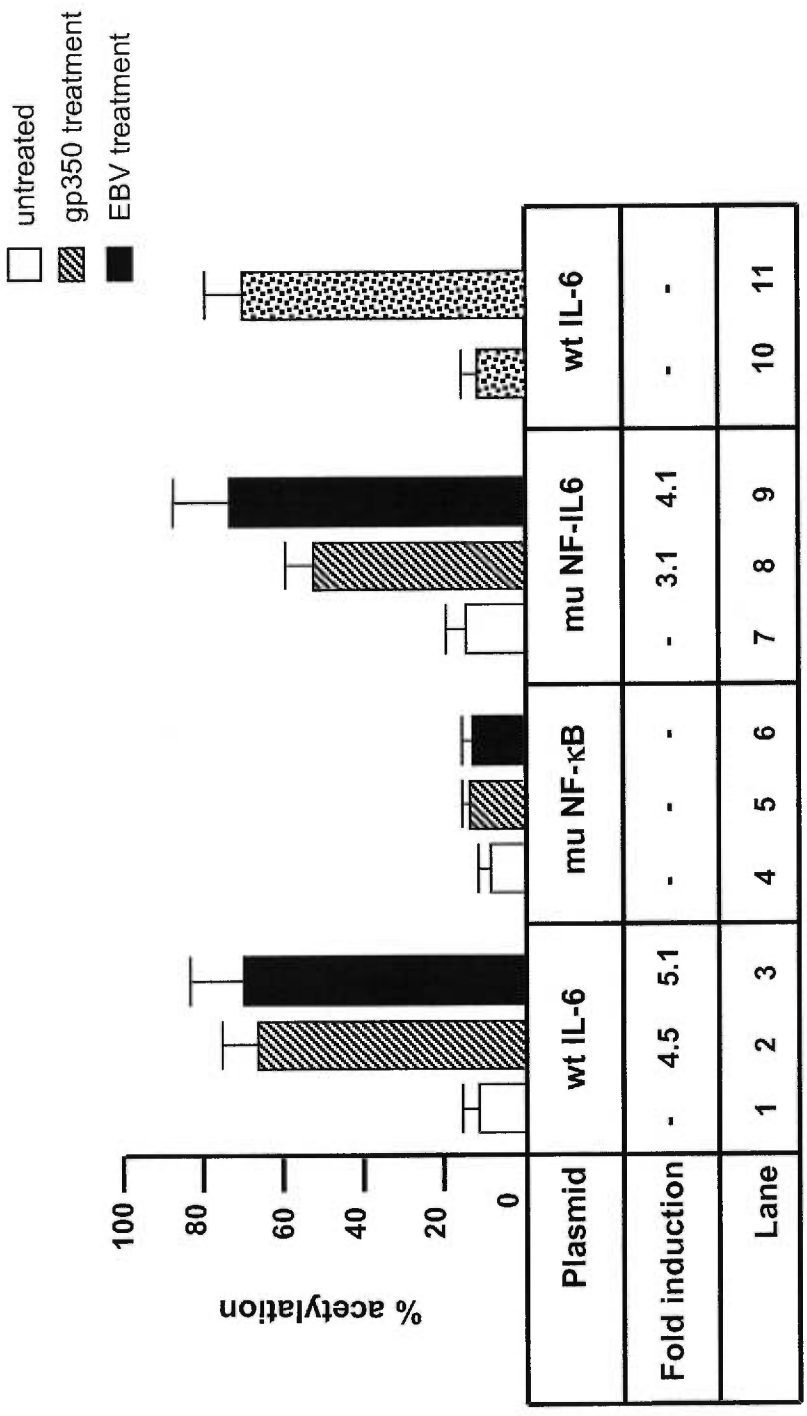
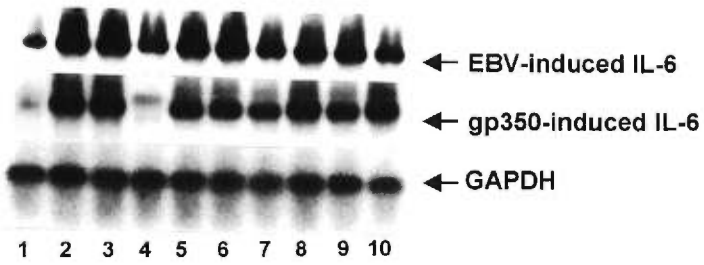

untreated
gp350 treatment
EBV treatment
% acetylation
100
80
60
40
20
0
Plasmid
wt IL-6
mu NF-κB
mu NF-IL6
wt IL-6
Fold induction
- 4.5 5.1
- - -
- 3.1 4.1
- -
Lane
1 2 3
4 5 6
7 8 9
10 11

Figure 7


EBV-induced IL-6
gp350-induced IL-6
GAPDH
1 2 3 4 5 6 7 8 9 10

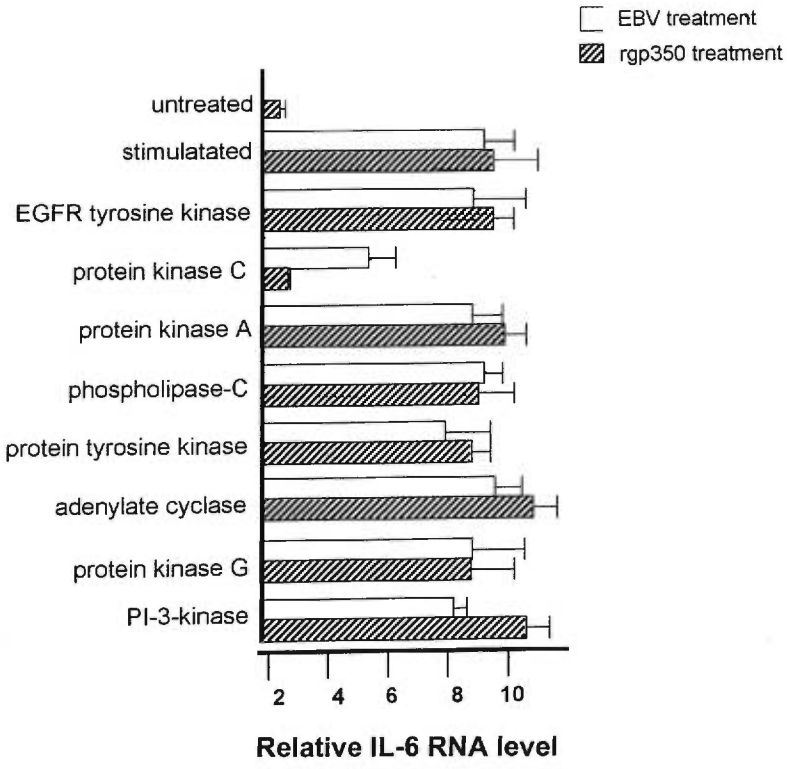

EBV treatment
rgp350 treatment
untreated
stimulatated
EGFR tyrosine kinase
protein kinase C
protein kinase A
phospholipase-C
protein tyrosine kinase
adenylate cyclase
protein kinase G
PI-3-kinase
2 4 6 8 10
Relative IL-6 RNA level

Figure 8

3.0 DISCUSSION

3.1. Differential Regulation of Cytokine Gene Expression during Virus Infection.

In response to invasive agents, cytokines and the immune system function in unison to remove the pathogen. Mor specific reviews describing viral infection and pathogenesis can be obtained elsewhere (Oldstone, 1996 and Spriggs, 1996) and a similar virus receptor review is also available (Haywood, 1994). In response to viral infection however, cytokines and other cellular processes may be differentially modulated. Infection of monocyte/macrophages with viruses such as vaccinia virus, HSV-1, -2, EBV, HHV-6, CMV, influenza virus, respiratory syncitial virus (RSV), Sendaï virus or HIV significantly alters cellular functions. In the following pages, an analysis of virus-cell interaction will be undertaken concentrating on the effects of virus on cytokine production. Emphasis will be placed on HIV and EBV since much more is known about these viral systems. Together, all these studies show that different viruses may differentially stimulate or repress certain cytokines in different cell systems.

The results presented in this thesis demonstrate that EBV and gp350 treated monocytic cells express increased levels of cytokines IL-1β and TNF-α mRNA and protein. Stimulation of cells resulted in increased cytokine gene transcription and translation, which was partially due to increased mRNA stability compared to TPA treated cells. In addition, the results demonstrate that gp350 interaction with CD21/CR2 induces binding of NF-κB proteins to both TNF-α and IL-1β-κB concensus sequences. Treatment of these cells with specific signal transduction inhibitors subsequent to gp350 stimulation demonstrated the involvement of PKC and PI-3-K dependent pathways. This inhibition is demonstrated at the level of IL-1β and TNF-α mRNA and protein production as well as decreased binding of NF-κB p50 and p65 subunits to both cytokine gene -κB consensus sequences.

Analysis using other viral models demonstrates that the results obtained in this EBV cell model are not unique. The interaction of virus and cell proteins is common and

often leads to aberrant viral/cellular gene expression and activity. In many cases, virus interaction with membrane receptors is sufficient to stimulate immune responses as it pertains to the production of cytokines.

3.2. Human Immunodeficiency Virus (HIV).

This section will deal specifically with HIV and its ability to modulate cytokine gene expression since a great deal of work involving this virus has been done. A recent review provides adequate information concerning the immunopathogenesis assoicated with HIV infection (Pantaleo and Fauci, 1995).

Early data found that the addition of mitogens to HIV-infected cell cultures produced elevated levels of various cytokines compared to equivalently stimulated non-HIV infected cells. In this context, these virally infected cells could be assumed to be primed relative to mitogen stimulation, as are Sendai virus treated cells. HIV studies using numerous monocytic and T-lymphoid cell lines determined that IL-1α, IL-1β, TNF-α, -β, IFN-α, IFN-β and IL-6 were produced in greater amounts in HIV infected cells (Folks et al., 1987, Breen et al., 1990, D'Addario et al., 1990, 1992, Roulston et al., 1992 and Yamato et al., 1990). These early studies determined that elevated cytokines were present in these cells, but did not examine the mechanisms underlying this phenomenon.

Roth (1990) found that PBMC isolated from HIV infected or AIDS patients were much more sensitive to vesicular stomatitis virus challenge *ex vivo*. Sensitivity in this case was demonstrated with increased transcription and translation of TNF-α and TNF-β. This report also found that IFN-α, while abundantly transcribed was not secreted but remained intracellularly associated with polyribosomes demonstrating differential post-transcriptional regulation of different cytokine genes. Vyakarnam (1991) found similar results when

analyzing TNF-α, -β and IFN-γ in LPS treated PBMC. Allen (1990), Honda (1990), Yamato (1990) and Merrill (1989, 1992) analyzed the expression of the IL-2 receptor, IL-6 and IL-1β transcription and translation in PBMC. Our results with EBV support the association between viral interaction with cell surface receptors and the activation or suppression of cytokine genes.

More recently, Yoo (1996) found increased IL-10 and IL-12 production from monocyte/macrophages and T-lymphocytes after HIV infection while other studies analyzing hematopoietic cytokines such as IL-6 and IL-8 were found to be elevated in primary human monocytes (Esser et al., 1996). Cytotoxic T-cell activity and release of TNF-α and TNF-β were also elevated in response to antigen in HIV infected blood mononuclear cells (Jassoy et al., 1993). These results indicate that antigenic stimulation of HIV infected cells leads to the production of significantly higher levels of cytokines further disrupting normal immune function. In addition to these reports, others demonstrate elevated levels of apoptosis in the CNS of HIV infected individuals (Shi et al., 1996) indicating that HIV may use several mechanisms to disrupt normal cellular function.

As with EBV, specific HIV related proteins are now known to mediate this altered cytokine production. Of HIV, the 15 kDa Tat protein interacts with stem-loop the structure at the 5'-end of HIV-RNA and *trans*-activates the expression of all sequences linked to the HIV-LTR (Sharp and Marciniak, 1989). The HIV-Tat protein will transactivate the TGF-β promoter in human B-lymphocytes and this activation occurs at the transcriptional and post-transcriptional level (Jagannadha-Sastry et al., 1990). More recent examination of HIV-Tat-TGF-β association shows that Tat interacts with a TAR-like element located at the 5'-end of the TGF-β promoter in both monocyte/macrophages and T-lymphocytes (Bunaguro et al., 1994). In this latter study, Tat was found to co-operate with the NF-κB and Sp1 cellular transcription factors to mediate this effect. Stimulation of cytokine gene synthesis does not require HIV per se as data shows that uninfected monocytes

transfected with HIV Tat will transcribe and translate elevated levels of IL-1α, IL-1β, IFN-γ, TNF-α, TNF-β, GM-CSF, IL-2 and neopterin (Rautonen et al., 1994).

The association of Tat and elevated cytokine levels is strengthened by evidence demonstrating that this viral protein not only stimulates Kaposi's sarcoma-derived cells *in vitro* (Ensoli et al., 1990), but that growth of these cells involves the secretion of high levels of IL-1β, basic fibroblast growth factor and other angiogenic agents (Ensoli et al., 1989). Tat's angio-proliferative properties involve selective activation of spindle cells, increases in vascular cell migration and degradation of basement membranes associated with Kaposi's sarcoma (Albini et al., 1995). In the context of Kaposi's sarcoma, HIV-Tat is now known to augment monocyte adhesion and chemotaxis by more than 10 fold (Lafrenie et al., 1996).

The effects of Tat are at least mediated by this viral protein`s ability to interact with cell surface integrins $\alpha_v\beta_1$ and $\alpha_v\beta_3$ (Albini et al., 1995). In addition, Tat is known to selectively stimulate phosphotidyl-inositol-specific-phospholipase-C in T-cell cultures and may mediate its effects through the CD28 component of the IL-2 receptor (Zauli et al. 1995a and Ott et al., 1997). The induction of this specific signal transduction pathway is rapid and results in the nuclear localization of Tat within 30 minutes of treatment. This viral protien is also capable of inducing apoptosis of $CD4^+$ T cells through activation of the HIV-LTR (Zauli et al., 1996). In addition, Tat will induce Bcl-2 and c-Fos gene expression in T-lymphocytes and PBMC and induce NF-κB and PKC in human neural astrocytes (Zauli et al., 1995b, Conant et al., 1996 and Giberllini et al., 1997). In the case of EBV treatment of peripheral blood cells, we found that virus selectively activated NF-κB through PKC and PI-3-K.

Another HIV protein shown to be active in immunomodulation is the surface ligand gp120. This protein, on its own, is capable of inducing TNF-α, IL-1β, GM-CSF and IL-6 in monocytes and Ig secretion from the B-lymphocytes of HIV-1 infected individuals (Clouse et al., 1991 and Rieckmann et al., 1991). Gp120 analysis has been extended to show that

IL-10, IL-2 and IFN-α_2 increase in PBMC (Ameglio et al., 1994). In synergy with IFN-γ, gp120 will induce IL-12 secretion in monocyte/macrophages (Fantuzzi et al., 1996) and stimulate nitric oxide release in fetal, neonatal and adult brain glial cell cultures (Koka et al., 1995). More specifically, this viral glycoprotein will also induce production of ERK (extracellular-regulated-kinase), MAPK (mitogen-activated-protein-kinase) and Janus kinase in T-lymphocytes (Jabado et al., 1997). In addition, gp120 will enhance ICAM-1 expression in human and rat astrocytes, astroglioma cell lines and microglia (Shrikant et al., 1996) and induce CMV production with the assistance of endogenous IL-8 (Capobianchi et al., 1997).

Like gp120, EBV gp350 is now known to activate the production of cytokines from AMM or from B-lymphocytes (Tanner et al., 1986). In the latter case, the stimulation of IL-6 was dependent on PKC and PTK acting almost immediately following treatment of cells. More recent results from our lab suggest that activation of IL-6 by EBV or gp350 in B-lymphocytes requires differential stimulation of multiple signal transduction pathways and NF-κB (D'Addario et al., mansucript submitted).

3.3. Herpesvirus Mediated Immunomodulation.

Herpesviruses exist in most cases in latent form in various tissues and in this mode are able to evade the host immune response. During viral infection a selective decrease in MHC class I mediated immune response commonly occurs due to the virus' ability to shut down most cellular protein translation.

In this context anti-HSV responses are more likely to not be $CD8^+$ MHC class I restricted but $CD4^+$ MHC class II restricted, diminishing the cytotoxic T lymphocyte response to virally infected cells (Koelle, et al., 1993). A HSV strain capable of modulating MHC class II expression following intraocular inoculation, HSV strain F will not only suppress expression of MHC class II, but will diminish the expression of IL-6 and IFN-γ while not affecting production of TNF-α or IL-1 (Lewandowski et al., 1993). While the mechanism involved in the latter case in not known, the EBV-BZLF2 gene product suppresses MHC class II expression through intracellular interaction, inhibiting its transport to the cell membrane from the golgi apparatus (Li et al., 1995).

CMV is capable of stimulating TGF-β production through its immediate early genes-1 and -2 (IE-1, and -2, Michelson et al., 1994). This induction occurs at the transcriptional level for both TGF-β and TNF-α and mutation of either CMV viral protein completely abrogates the inductive capabilities on both the TGF-β and TNF-α promoters (Geist et al., 1994). Interestingly CMV encodes an immediate early gene (US3) capable of insertion into the cellular rough endoplasmic reticulum (rER) and retains antigens or MHC class I molecules (Ahn et al., 1996). In this case, CMV could inhibit cell surface expression of its own viral antigens in the context of MHC, thereby minimizing or eliminating an immune response.

In the case of CMV, decreased MHC class I expression occurs through its UL18 gene (Yamashita et a., 1994). The UL18 gene product was found to not only inhibit the transport of MHC class I proteins to the cell surface, but enhanced MHC class I protein

degradation in the rER. This mechanism aids CMV survival by decreasing the expression of cell surface markers necessary for immune-mediated lysis of virally infected cells.

The CMV-UL111A ORF codes for a protein termed *MTRII*. This viral protein, when mutated will no longer allow for CMV-induced cellular transformation. Its presence in papilloma-associated dysplasias suggests this CMV oncoprotein may aid the development or progression of cervical tumors. Whatever its role in these particular malignancies, the *MTRII* CMV oncoprotein is capable of interacting with and inhibiting p53 mediated transcriptional activation of cellular genes within 12 hr of infection (Muralidhar et al., 1996). The expression of such herpesvirus proteins demonstrates that these viruses have evolved to survive in a complex manner.

HSV-1 is known to infect neural cells and remain latent for extended periods of time. Following ocular infection of mice, Halford (1996) found that 100% of cells produced IL-2, IL-10, TNF-α, IFN-γ, and RANTES mRNA and protein; this cytokine synthesis remained active up to 45 days after initial innoculation. The authors found that HSV-1 remained in the trigeminal ganglion even after mononuclear cell infiltrates had left the site of infection. In addition, cytokine production was still evident following the outward migration of mononuclear cells. Antigens presenting dendritic cells are also reactive to HSV-1 challenge. Treatment of these cells with HSV-1 resulted in decreased expression of CD3, CD14 and CD19 while CD4 and CD83 cell surface expression was augmented (Ghanekar et al., 1996). The authors also found that IFN-α, IL-1α, IL-1β, IL-6, IL-10, IL-12, GM-CSF and TNF-α were all stimulated within 4 hr of HSV-1 addition to cell cultures. These results show that in addition to altering the expression of cytokines, herpesviruses can also deregulate the expression of cell surface markers so that cells respond differently to cellular challenge.

As with HIV-gp120, the HSV-gB2, -gD2 or -gG2 viral antigens were analyzed by Carmack (1996) for their ability to deregulate cytokine synthesis. The authors found IL-2

and IFN-γ to be preferentially stimulated in both seropositive and seronegative patients. Interestingly however, the seropositive patients consistently produced slightly higher levels of cytokines, which again may indicate priming of cells to HSV-1 and -2 antigens.

Gosselin (1991, 1992a and 1992b) found that HHV-6 could stimulate IL-1β, while HSV and EBV did not, and conversely that IL-6 and TNF-α were significantly repressed by HHV-6 and EBV, while HSV addition was refractory. These studies were all undertaken in purified mononuclear cells from healthy patients and our studies further confirm that interaction of EBV-gp350 is sufficient to induce transcription and translation of these cytokine genes (D'Addario et al., 1999, and submitted).

Modulation of cytokine gene expression by herpesvirus may also be explained by recent studies showing that certain viral genes are homologous to cellular genes with known immunomodulatory function. The EBV-BCRF1 protein is homologous to hIL-10 and as such mediates many of the same effects as its human counterpart. Among its activities is the suppression of cytokine synthesis from virally infected cells and its ability to stimulate T-lymphocyte proliferation (Moore et al., 1993). Its expression druing EBV infection is unclear but evidence now indicates that it may not be necessary for virus survival as its elimination from the viral genome does not disturb EBV infectivity (Swaminathan et al., 1993).

All these reports demonstrate that infectious viruses have found a mechanism to evade or alter the immune response. With the discovery of viral gene homologs to cellular proteins, these agents contain an arememtarium capable of modulating an immune response generated against them.

3.4 EBV proteins Implicated in Cellular Gene Regulation.

3.4.1. EBV Latent Membrane Protein-1 (LMP1).

EBV-LMP-1 is expressed in lymphoblastoid cell lines transformed *in vitro*, in EBV-associated diseases such as infectious mononucleosis, in preneoplastic lesions of NPC and in EBV-associated Hodgkin's disease. Expression of LMP-1 in different rodent cell lines induces immortalization, while certain mutant derivatives do not (Kieff, 1996).

LMP-1 is a membrane bound protein that extends through the cellular membrane six times and acts as a growth factor receptor. Its introduction into B-lymphocytes induces phenotypic changes characteristic of activated lymphocytes, including induction of DNA synthesis, increase in cell size, upregulation of cell surface activation markers and adhesion markers and induction of homotypic cell adhesion (Kieff, 1996). Its presence on the cytoplasmic membrane is transient; it is proteolytically degraded and hence may not appear to be expressed at all.

Biochemical analysis has demonstrated that its carboxy terminal (CT) area is essential for transformation, the first 54 amino acid CT residues alone are sufficient for B-lymphocyte transformation (Kieff, 1996). At its CT area, LMP-1 is phosphorylated at serine and threonine residues and is known to associate with many intracellular proteins.

Transient expression of LMP-1 containing plasmids will activate cellular genes responsive to the NF-κB transcription factor (Herrero et al., 1995 and Mitchell and Sugden, 1995). These studies found that this activity was reproducible using mutant LMP-1 proteins that were unable to induce transformation demonstrating that these two activities may be dissociable in this viral protein. LMP-1 mediated NF-κB activation may play a role in the activation of the *Bcl-2* oncogene (Rowe et al., 1994 and Wang et al., 1996). The induction of *Bcl-2* was accompanied by the increased expression of cell surface ICAM-1, so the relative effects of LMP-1 may be time dependent. Wang (1996) showed that induction of

Bcl-2 may require prolonged infection, a rapid transient initial activation of a Bcl-2 homolog, Mcl-1 in B cells may be necessary prior to Bcl-2 production. These results demonstrate that LMP-1 may potentially induce cellular activities through NF-κB and may mediate its transforming effects through the stimulation of Bcl-2.

Recently, the first 50 CT LMP-1 amino acid residues were found to be associated with the TNF receptor family of proteins, TRAFs (Mosailos et al., 1995 and Kaye et al., 1996). Activation of NF-κB may result from LMPs association with the intracellular component of the TNF-α receptor and TRAF1, TRAF2 and TRAF3 (TNF-receptor associated factors, Devergne et al., 1996).

TRAFs are cytoplasmic proteins known to interact with the intracellular component of CD40 and TNF-α receptors (both are components of the TNF-α receptor superfamily). Devergne et al (1996) found *in vitro* association of TRAFs with LMP and found that this interaction occurs at the level of LCL cell membranes. In addition to its ability to activate NF-κB through TRAFs, LMP through the TRAFs, may induce other effects such as B-lymphocyte growth transformation (Izumi et al., 1997) and activation of latent HIV specifically through TRAF2 and NF-κB (Tsitsikov et al., 1997).

Another cellular oncogene whose expression is modulated by LMP-1 is p53. This anti-oncogene is known to be mutated in over 50% of all human malignancies; its association with EBV related disorders demonstrates it's modulation in Burkitt's lymphoma, AIDS associated lymphomas, Hodgkin's disease Reed-Sternberg cells and NPC (Alero Thomas et al., 1991). Following EBV infection of resting human B-cells, p53 expression was found to increase by 10 fold over 5 days (Chen and Cooper, 1996). The exact mechanism controlling p53 expression is not yet characterized but transient transfection studies using specific viral genes could easily determine which are associated with this effect and in what sequence. The studies using LMP-1 demonstrate that this viral protein is capable of affecting cellular functions through direct or indirect interaction with cellular

proteins. While the above mentioned affects of LMP1 do not demonstrate affects on cellular cytokine genes, experiments of this sort would certainly determine if such an association exists and whether this viral protein is capable of affecting this class of genes.

3.4.2. Epstein-Barr Nuclear Antigen -1.

EBNA1 is expressed in all EBV infected cells, and binds DNA as a homo- or heterodimer. It has an established role in EBV replication and maintenance of the episomal form of the viral genome, mediating its effects by binding to the upstream portions of EBV oriP and Qp (Wilson et al., 1996 and Kieff, 1996).

While LMPs association with NF-κB is through its ability to activate a number of cellular genes via this transcription factor family, EBNA1 plays a significant role during infection. EBNA1 augment CD21 and CD23 cell surface expression on EBV-infected B-lymphocytes and stimulate LMP1, HIV-1 LTR and IL-10 gene expression (Kieff, 1996). Its lack of expression in the P3HR-1 EBV strain does not permit this strain to transform B-cells *in vitro* (Nakagomi et al., 1994). These viral gene products associate with the nucleus of infected cells and may function as specific transactivators of heterologous cellular and viral genes (Kieff, 1996).

Through its association with HIV-Tat, EBNA1 is known to activate the HIV-1 LTR through specific subunits of the NF-κB family of proteins (Scala et al., 1993). To this end, Paine (1995) have shown that EBNA1 is capable of activating transcription of genes p50 and p52, two proteins part of the NF-κB family.

In addition to these studies, activation of latent EBV may be partially mediated by EBNA1 induced activation of interferon response factors (IRFs). The IRF1/IRF2 family of proteins bind to upstream sequences of the IFN-β promoter genes (Schaefer, et al., 1997),

and are responsible for virus mediated activation of this cytokines. Data now shows that targetted mutation of the upstream IRF1/IRF2 sequences of the EBV-Qp promoter disrupts viral stimulation from latency, and that EBNA1 may be activating these proteins to act at the Qp promoter site.

The association of EBV infection with high levels of cellular transcription factors is not surprising since EBV proteins are capable of stimulating these proteins' functions. To this end, Hodgkin/Reed-Sternberg cells in culture contain much higher levels of Oct-2, and NF-κB cellular transcription factors (Bargou et al., 1996). Although the role of any particular EBV protein in this system was not analyzed, LMP1 or EBNA1 were most likely involved. This demonstates that EBNA1 is not only capable of activating cellular genes, but may be regulating those cellular proteins involved in transcriptional control.

3.4.3 EBV BCRF1 (viral interleukin-10, vIL-10).

The human interleukin-10 protein shares up to 84% amino acid sequence homology with its viral counterpart viral interleukin-10 (vIL-10, Moore et al., 1993). Coded for by the EBV *BCFR1* gene, vIL-10 shares many of the same functions as its hIL-10 (Moore et al., 1993). Originally thought to be expressed only during the late phase of the lytic cycle of EBV infection, vIL-10 may play a pivotal role in B-cell transformation as data now shows it to be necessary for this process (Miyazaki et al., 1993). Its effects, compared to its human homologue, are somewhat reduced. While vIL-10 can activate B cell MHC class II and suppress IFN-γ and GM-CSF induced macrophage activation, it does so less efficiently. Viral IL-10 has the same effects as hIL-10 and its expression during the lytic cycle may provide EBV with a window of immuno-suppression but its complete role during latent or lytic infection is not yet completely understood. As mentioned above, while some

data indicates that it is necessary for virus induced transformation (Miyazaki et al., 1993), other data indicates that its loss in EBV-BCFR1 negative mutants, has no effect on viral latency (Swaminathan et al., 1993). Although its role in the EBV life cycle may not be clear, its effects on the immune system are better characterized and show that it strongly suppresses many critical immune activities.

Inhibition of cytokine production by T helper cells and monocyte/macrophages can be mediated by hIL-10 and less effectively by vIL-10. Cells stimulated with various mitogens can be inhibited from secreting IFN-γ, TNF-α, -β and GM-CSF by exogenous addition of hIL-10 (Moore et al., 1993). Human IL-10 is potent at inhibiting antigen presenting cell induced T cell proliferation through the decreased expression of cell surface MHC class II (de Waal-Malefyt et al., 1991). Langerhan cells induced with GM-CSF can also be inhibited from presenting antigen through pre-incubation with hIL-10 and in this context may delay a delayed type hypersensitivity response (Beissert et al., 1995). In addition to MHC class II, hIL-10 is also known to suppress GM-CSF/IL-4 induced expression of CD1 molecules on T-lymphocytes (Thomssen et al., 1995).

HIV infection can modulate the expression of IL-10 mRNA and protein from PBMC and in these cases increased IL-10 correlated with increased HIV burden, while addition of anti-IL10 mAb could reverse such viral loads (Clerici et al., 1994). Kootstra (1994) and Saville (1994) both found that in tissue culture, exogenous addition of hIL-10 to HIV infected monocyte/macrophages, but not T-lymphocytes, resulted in decreased virus production. This regulation was not due to decreased activation of an HIV-LTR-CAT construct or decreased cell viability, but the authors suggest that it may be due to diminished viral assembly in the cytoplasm of infected cells.

Recent evidence using viral models indicate that IL-10 may work along side TNF-α to activate HIV from latently infected monocyte/macrophages. Finnegan (1996) found that while IL-10 on its own can down-regulate HIV in monocyte/macrophages, concomitant

addition of this cytokine will reactivate latent virus to a greater level than TNF-α alone. The authors also demonstrate that while TNF-α alone can augment TNF-α receptor expression, hIL-10 and TNF-α together do not produce a similar effect. However, when added together, TNF-α and IL-10 enhanced levels of AP-1 and NF-κB transcription factor binding to the HIV-LTR, produced higher levels of CAT expression via the HIV-LTR CAT plasmid and increased HIV steady-state RNA levels (Finnegan et al., 1996). Interestingly, while IL-10 is known to suppress HIV expression, the HIV transactivator of transcription (Tat) has been shown to stimulate IL-10 production in these infected cells (Masood et al., 1994). Since the Tat protein is known to stimulate virus replication, its ability to stimulate IL-10 argues toward another level of HIV regulation that has not yet been characterized. As mentioned earlier, NF-κB in T-lymphocytes is inhibited by IL-10 even in the presence of a CD3 activation signal (Fiammetto-Romano et al., 1996), so clearly cell specific factors are involved in the regulation that is mediated by IL-10.

IL-10's ability to modulate immune activities is also shown by its inhibitory effects on IL-4 and GM-CSF induced generation of dendritic cells from peripheral blood cells (Beulens et al., 1997). This article shows that dendritic cells normally differentiated from PBMC via IL-4/GM-CSF stimulation are rerouted toward a more macrophage like phenotype when treated with hIL-10. Mature dendritic cells stimulated with hIL-10 also have enhanced antigen uptake capabilities, decreased allo-stimulatory capacity and contrary to monocyte/macrophages will augment HLA class II expression (Morel et al., 1997). These studies suggest that while hIL-10 will suppress immune activities in monocyte/macrophages, hIL-10 augments dendritic cell mediated antigen presentation and receptor expression.

Human IL-10 is also known to modulate a number of oncogenic activities. Its expression will suppress spontaneous GM-CSF production from peripheral blood and bone marrow mononuclear cells in patients with chronic myelomonocytic leukemia

(CMML). In this case, IL-10 decreased spontaneous growth of CMML myeloid cells in over 90% of patients tested (Geissler et al., 1996). Similar effects are observed when tumor metastasis was found to decrease through an IL-10 induced natural killer (NK) cell activation process. After intravenous injection of melanoma cells into mice, Zheng (1996) found that IL-10 inhibited tumor expansion via recruitment of NK cells to the tumor nidus. Similarly, IL-10 is to be expressed at higher levels in basal cells of benign squamous cell carcinomas. In this case, decreased expression of IL-10 was found to diminish tumor infiltrating lymphocyte recruitment, hinting that IL-10 may provide a pathway for neoplastic cells to avoid T-cell mediated cytotoxic responses (Kim et al., 1995).

Although the above-mentioned effects of IL-10 concentrate on hIL-10, the assumption is that the viral homolog can modulate similar activities, only to a lesser extent. This assumption is supported by evidence demonstrating that vIL-10 can also suppress local T cell mediated tumor rejection mechanisms in mice expressing melanomas (Suzuki et al., 1995). The authors show that mice containing either melanomas or sarcomas containing retroviral vectors expressing vIL-10 would succumb at greater frequency compared to those mice provided with an anti-IL-10mAb.

3.5. Cytokine Gene Regulation Mediated by Membrane Receptors and CDs.

The clusters of differentiation (CDs) are found on all cells and provide them with unique markers for identification. These cellular receptors mediate many activities ranging from antigen recognition, cellular differentiation and activation and stimuli for any number of other cellular activities.

As mentioned above, CD21/CR2 in cooperation with CD19 may mediate the activation of cytokine genes in response to EBV-gp350. A growing amount of data now demonstrates how extracellular signals, through the CDs, traverse the cellular membrane and modulate cellular function. A review will be provided in this section demonstrating how particular CDs are able to modulate cellular activities. In addition, cytokine responsive genes are now being well characterized due to a number of techniques (Beadling and Smith, 1994) which analyze for specific cellular gene activation following treatment with specific mitogens. A complete review of cytokine receptors is provided elsewhere (Ihle et al., 1995); what follows is a review specific to cytokines presented in this thesis.

Cytokines induce very different responses when cells are treated with distinct stimuli. In the same manner, a similar signal may produce an alternate response when different cells are affected. The cytokine receptor superfamily encompasses a multitude of different receptors, arguing against a common ancestry. Sharing common extracellular structural domains, the differences in their intracellular responses will be mediated by differential activation of intracellular secondary signal transduction mechanisms.

Although originally described as a receptor involved in activation or apoptosis, CD40-ligand interaction on B-lymphocytes will promote proliferation and differentiation, the survival of germinal center B cells and Ig class switching (Banchereau et al., 1994). A member of the TNF receptor superfamily, CD40 is also expressed on basal epithelial cells, carcinomas and transformed cell lines.

Engagement of CD40 with ligand will induce IL-12 mRNA and protein production in

macrophages (Kato et al., 1996); in B cells, this receptor-ligand interaction will induce other signal transduction pathways. Induction of IL-6 mediated by NF-κB is a hallmark of CD40 receptor engagement in nonhematopoietic cells (Hess et al., 1995). Sutherland (1996) found that crosslinking of CD40 will specifically stimulate c-Jun N-terminal kinases (JNKs), while not enhancing extracellular signal-regulated kinases (ERKs) and p38 mitogen activated kinases (p38 MAPKs). In contrast, ligation of the B cell antigen receptor would only activate JNK and ERK, without affecting p38 MAPK. Selective induction of signal transduction pathways will selectively activate transcription factors and cellular genes. With the induction of ERK, cellular and nuclear serum response factors will activate the *c-fos* and *c-jun* genes, while MAPK will not (Angel and Karin, 1991).

Other reports also show the activation of genes proceeding ligation of membrane CDs. Engagement of CD30, an immunological marker expressed in Hodgkin's lymphoma, will stimulate NF-κB (Duckett et al., 1997). The authors did not analyze cellular gene transcriptional activation, but NF-κB induction has previously been shown to activate latent HIV. Similarly, activation of neutrophil CD63 will stimulate tyrosine kinase activity and a lesser amount of threonine and serine kinase activity (Skubitz et al., 1996).

CD28, a molecule involved in T-cell stimulation (Linsley and Ledbetter, 1993), will also selectively activate NF-κB. Although CD28 is known to be involved in the activation of IL-2, its mechanism is unknown. Recent data seems to indicate that the NF-κB p50/RelA complex may be mediating this transcriptional activation (Harhaj, et al., 1996). The authors show that a biphasic induction of NF-κB components occurs over a 4 hr period; different combinations of p50-RelA-c-Rel will be activated and bind to the CD28-responsive element at different times with different affinities. To add to this complexity, Edmead (1996) shows that ligation of CD28 will also activate AP-1 through both the PI-3-K and acidic sphingomyelinase signal transduction pathways.

Lastly, and most importantly as it pertains to the work presented in this thesis,

engagement of another member of the complement family is now also known to stimulate cytokine genes. C3a and C3a desArg, upon release as degradation products of complement C3, will induce IL-1β and TNF-α synthesis in PBMC (Takabayashi et al., 1996). Interestingly, the activation of these two cytokine genes differs significantly from adherent to nonadherent PBMC. The authors demonstrate that while nonadherent cells actually decrease secretion of both cytokines, adherent cells augment IL-1β by ~18% and TNF-α by ~27%. In addition, our results demonstrate that activation of these two genes in PBMC by CD21 is at least partially mediated by the NF-κB family of transcription factors.

4.0 Conclusion.

Taken together, our results demonstrate that cytokines IL-1β, IL-6 and TNF-α can be transcriptionally and translationally elevated during the transient interaction of EBV or gp350 with the cell surface receptor CR2/CD21. This increased transcription and translation appears to be transient and may result in a more stable mRNA form. As mentioned above, IL-1, IL-6 and TNF are factors known to activate and are themselves activated by the NF-κB family of transcription factors. This complex interplay between EBV viral genes, cellular cytokine genes and NF-κB may potentially lead to a sequence of events culminating in immuno-dysregulation. TNF-α and IL-1β at high levels are both known to be very toxic and may potentiate pathology; our observation that both cytokines are produced at extremely high levels by antigenically stimulated PBMC indicates that these factors may be critically involved in EBV induced pathology.

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