Cytotoxic T-Lymphocyte Responses During Acute Epstein-Barr Virus Infection

Brian L. Beaulieu
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A Dissertation Presented

By

Brian L. Beaulieu

Submitted to the faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirement for the degree of

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May 13, 1996
CYTOTOXIC T-LYMPHOCYTE RESPONSES DURING ACUTE EPSTEIN-BARR VIRUS INFECTION

A Dissertation Presented

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Program in Immunology/Virology

May 13th, 1996
Dedicated to

my parents
Leo and Constance Beaulieu

my siblings
Brenda, Deborah, Kevin, David, Gary, Scott

and especially
my best friend and understanding partner
Sandra Delphin
ACKNOWLEDGEMENTS

I thank my mentor John Sullivan M.D. for providing me a superb role model, and the opportunity to share in his scientific endeavors. I would also like to thank the members of my committee, Raymond Welsh Ph.D., Francis Ennis M.D., Dale Greiner Ph.D., Mario Stevenson Ph.D., and Fred Wang M.D.

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I would also like to thank the American Heart Association for providing me with one year of financial support through the Medical Student Research Fellowship Program.
ABSTRACT

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus which causes acute infectious mononucleosis and is etiologically associated with malignant lymphoproliferative disorders including Burkitt's lymphoma, nasopharyngeal carcinoma, B-cell lymphomas in immunocompromised hosts, Hodgkin's disease, T cell lymphomas, and smooth muscle tumors in allograft recipients. The medical significance of EBV is underscored by its potent growth transforming effects on human B-lymphocytes in-vitro and the potentially oncogenic consequences of infection in-vivo. The majority of EBV-associated malignancies occur in the setting of chronic infection and strong virus-specific humoral immunity, suggesting that cellular immunity is primarily responsible for preventing the outgrowth of EBV-transformed B cells in-vivo. Similarly, primary EBV infection in adolescents and adults stimulates an intense cytotoxic-T-lymphocyte (CTL) response which coincides with a marked reduction in the number of infected B cells in the peripheral blood. Evidence of previous EBV infection can be confirmed by the presence of EBV-specific, HLA-restricted memory T cells in the peripheral blood which inhibit the outgrowth of newly EBV-transformed B cells and efficiently lyse established autologous B-lymphoblastoid cell lines.

Worldwide, EBV is responsible for substantial morbidity, comparable to measles, mumps and hepatitis virus, for which vaccines exists. Accordingly, the potential public health impact of an EBV vaccine has reinforced our efforts to identify the immunodominant virus-encoded T-cell epitopes which stimulate naive CTL effectors during acute infection and maintain memory CTL surveillance during convalescence. The EBV-encoded antigens against which the memory CTL response is directed have been partially defined, and include most of the EBV latent proteins (EBNA-2, 3a, 3b, 3c, LP, and LMP-1, 2a, 2b) consistently expressed by in-vitro EBV-transformed B lymphocytes (type-III latency). Importantly, all EBV-associated malignancies express EBNA-1, and as yet no EBNA-1-specific memory CTL have been
convincingly demonstrated. Additionally, many EBV-specific CTL lines and clones have been described which do not recognize any of the known latent proteins or other EBV protein antigens tested thus far. Thus while much is known about CTL-mediated immunity against EBV, our knowledge of EBV-derived CTL epitopes remains incomplete. In contrast to the EBV-specific memory CTL response, very little is known about the source of viral epitopes recognized during the primary CTL response to EBV. In this regard, acute infectious mononucleosis represents an ideal model system to study virus-specific, cell-mediated immunity. Acute IM is a self-limited illness characterized by the appearance of "atypical" lymphocytes (CD3+/CD8+/HLADR+), including both virus-specific and alloreactive CTL, which undoubtedly contribute to virus elimination and provide CTL precursors for life-long immunity to EBV.

Like other herpesvirus, EBV can undergo either lytic or latent cycle replication. During primary EBV infection many lytic cycle genes are expressed which are likely responsible for stimulating the intense cellular immune response associated with acute infectious mononucleosis. During convalescence a minor population of circulating B cells remain latently infected, harbor multiple EBV episomes, and express only EBNA-1 and possibly LMP-2a (type-I latency). Thus, latency type-I infected B cells in-vivo express a much more restricted spectrum of latent proteins and are therefore not subject to elimination by the same virus-specific CTL as are type-III EBV latently infected cells. Accordingly, many mechanisms have been proposed to explain EBV persistence including; restricted expression of EBV latent genes, reduced levels of cellular adhesion molecules, downregulation of MHC class-I molecules, absence of EBNA-1 T-cell-epitopes, and most recently, EBNA-1-mediated inhibition of antigen processing. While these mechanisms may contribute to ineffective T cell surveillance against latency type-I EBV-infected cells, B cells expressing the full spectrum of latent proteins (type-III) also exist transiently in vivo and maintain detectable humoral and CTL responses to most latent proteins.

Our first goal was to identify the virus-encoded immunodominant antigens recognized by in-vivo activated MHC class-I restricted CTL isolated from college students experiencing
primary EBV infection, manifested as acute IM. Following a prodromal period of several weeks, newly EBV infected patients present with signs and symptoms of acute IM, including elevated numbers of activated CD8+ T cells in their peripheral blood, many of which, like memory CTL, are EBV-specific and HLA-restricted. In order to address the issue of EBV persistence and the immune control of EBV-induced lymphoproliferation, we also studied the long-term EBV-specific memory CTL response in these same individuals.

Blood from acute IM patients and healthy EBV seropositive donors served as a source of peripheral blood lymphocytes to generate bulk CTL cultures and autologous target cells. The infecting strain of EBV was determined for each patient by DNA-PCR amplification of virus from saliva. Lymphocytes were isolated from whole blood by Ficoll-Paque density centrifugation and T- and B-cell enriched populations were obtained by AET-sheep red cell rosette selection. Autologous B cell blasts served as a source of target cells and recombinant vaccinia virus constructs were used to introduce individual EBV latent genes into target cells. Expression of individual EBV genes in target cells was confirmed by both western blot and immunofluorescence. Primary CTL responses to EBV were evaluated in standard 51Cr release assays using freshly isolated, T-cell enriched PBL from acute IM patients as effector cells. EBV-specific memory CTL responses were evaluated with bulk CTL culture generated by in-vitro restimulation with autologous B-LCLs. FACS analyses were routinely performed on bulk cultures of effector CTL populations in order to more clearly characterize their phenotype. Lastly, monoclonal antibody blocking studies and cold target competition assays were performed in order to accurately identify the viral antigen and MHC components responsible for target cell recognition.

Our results based upon evaluation of 35 acute IM patients and 32 convalescent patients demonstrate that the virus-specific primary CTL response is broadly directed against the full spectrum of latent proteins, including EBNA1 and the viral coat glycoprotein gp350, while the memory CTL response, which essentially lacks EBNA1 reactivity, is directed primarily
against the EBNA 3 family of proteins (3A, 3B, 3C). Importantly, the immunodominant response by both primary and memory CTL was directed against the EBNA3 proteins.

CTL from 7 of the 35 acute IM patients evaluated recognized EBNA1 expressing targets, and in 4 of these 7 patients, EBNA1 was an immunodominant antigen. Similarly, CTL from 7 of 35 acute IM patients recognized gp350 transfected targets, while no gp350-specific memory CTL responses were observed.

While the phenotype of in-vivo primed CTL effectors were CD8+/HLA-DR+/CD11b+, the major subpopulation of memory CTL were CD8+/HLA-DR+/CD11b−. The CD11b “memory marker” reached peaked levels on the first sample day for all patients and gradually declined to baseline levels over a period of several months. In contrast, the CD11b marker was quickly shed from in vitro propagated CTL, over a period of 5-10 days.

Target cell lysis by in-vivo activated CTL was almost completely blocked by antibody directed against class-I molecules (BBM.1), whereas the effect of blocking target cell lysis by anti-CD8 mAb varied between 40-75%. These findings are consistent with an absolute need for class-I restricted antigen presentation, and imply that CD8 was variably required, likely for the lower affinity TCR/Ag combinations. Cell lysis mediated by in-vitro-restimulated memory CTL was also largely inhibited by anti-class-I mAb, while anti-CD8 mAb was only mild/moderately effective in blocking target cell lysis, in keeping with the concept that memory CTL bear higher avidity TCR which can recognize antigen independent of CD8.

Our detection of only one EBNA1-specific memory CTL response among the 32 patients tested supports the theory that latently infected B cells in-vivo, expressing only EBNA1, escape CTL recognition and thus might serve as a reservoir for viral persistence and/or reactivation. The rare ability to detect an EBNA1-specific memory CTL responses remains a relatively unexplained phenomenon and may involve a number of tolerizing mechanisms including the induction of anergy by presentation of EBNA-1 in the absence of costimulation, clonal deletion of low affinity T cells, the absence of dominant T cell epitopes within EBNA1 or
a result of the recently described inhibiting properties of EBNA-1 on antigen processing and presentation.

Alternatively, the absence of detectable EBNA1-specific memory CTL may be the result of insufficient or inappropriate restimulation of memory CTL in vitro. We addressed this possibility by attempting to selectively restimulate and expand EBNA1-specific CTL from acute IM patients by using EBNA1 expressing B cells blasts as a stimulus. Effector cells generated in this manner killed target cells in an MHC class-I restricted manner but were specific for an unspecified vaccinia antigen. Interestingly, the phenotype of the effector cells was predominantly CD3+/CD4-/CD8-/γδ T cells.

In summary, our findings suggest that a multitude of previously unrecognized, EBV-specific CTL are present in the peripheral blood during acute IM, and include EBNA-1-specific CTL. The importance of accurately defining the in-vivo immune response to EBV is underscored by the ever-growing list of EBV associated malignancies. In addition to providing insights into the oncogenesis and potential treatment of NPC, a newly described link between precursor lesions and EBV infection raises the possibility that heightened immunity to EBV or EBV-infected cells may prevent the development of NPC. An obvious expectation would include extension of such knowledge to other EBV associated malignancies such as B and T cell lymphomas, Hodgkin's lymphomas, and smooth muscle tumors. First however, existing gaps in knowledge regarding the immune response to EBV and EBV-associated malignancies must be closed. Details about the viral gene products which are involved in stimulating a broadly protective, virus-specific immune response in a large number of individuals is fundamental to the design of an effective EBV vaccine. Since the presence of activated CD8+ T cells correlates with the rapid decline of EBV infected B cells in the peripheral blood, a concise description of the EBV-specific CTL response in the setting of acute infection will be necessary for the rational design of an effective acute IM vaccine. Increased understanding of viral escape mechanisms is also likely to contribute to therapeutic modalities to treat autoimmune disorders.
CHAPTER I. INTRODUCTION

A. History of Epstein-Barr Virus.
   1. Discovery and Classification of EBV.
   2. Virus & Genome Structure.
   3. Type & Strain Variations.
   4. Host Range & Virus Receptor.
   5. Stages of Infection.
      a. EBV adsorption, penetration, uncoating.
      b. Virus expression in latent infection.
   7. EBV DNA Persistence in Latency.
   8. Lytic Infection / Virus Replication.

B. Experimental Models of EBV Infection.

C. Clinical Manifestations & Immunobiology of EBV Infection.
1. Epidemiology.
2. Diagnosis.
3. Pathology.

D. Acute Infectious Mononucleosis.
   1. Clinical Manifestations.
   2. Humoral Immune Response to EBV Infection.
   3. Induction of EBV-specific CTL.
   4. Apoptosis of Activated CTL During Acute Infectious Mononucleosis.

E. Lymphoproliferative Diseases.
   1. X-linked Lymphoproliferative Syndrome.
   2. Primary Immunodeficiency Diseases.
   3. Burkitt’s Lymphoma.
   7. EBV Associated T Cell Lymphomas.

F. Non-Lymphoid EBV Diseases.
   1. Nasopharyngeal Carcinoma.
   2. Smooth Muscle Tumors/Aids/OHL/Liver Transplantation.

G. MHC Class-I-Restricted, EBV-Specific CTL.
   1. Phenotype of Effector Cells.
   2. Latent Proteins Recognized by CD8+ Memory CTL.

H. EBV Vaccines.
   1. Identification of Potential Vaccine Antigens.
   2. Studies with gp350-Based Vaccines.
   3. Clinical Implications of an EBV Vaccine.
CHAPTER II. MATERIALS AND METHODS

A. Experimental design.

B. Patients.
   1. EBV serology.
   2. EBV strain typing by DNA PCR amplification.
   3. MHC Class-I tissue typing.

C. Isolation of lymphocytes.
   1. SRBC-rosette separation of T- and B-lymphocytes.

D. Preparation of target cells.
   1. Generation of B cell blasts.
   2. Generation of B lymphoblastiod cell lines (B-LCLs).
   3. Generation of HTLV-1 transformed T-cell lines.
   4. Generation of primary human fibroblast lines.

E. Growth of vaccinia virus stocks.
   2. Sucrose gradient purification of vaccinia virus stocks.
   3. Titration of vaccinia virus stocks.

F. Expression of recombinant vaccinia-EBV constructs in target cells.
   1. Immunofluorescent detection of EBV nuclear antigens.
   2. Immunofluorescent detection of EBV latent membrane protein 1.
   3. Immunofluorescent detection of gp350/220.
   4. Immunoblot detection of EBV nuclear antigens.
   5. Immunoblot detection of EBV latent membrane protein 1.

G. Preparation of Effector Cells.
   1. Natural Killer cell depletions.
   2. Generation of EBV-specific memory CTL cultures.
3. Cytotoxic T lymphocytes from acute IM patients.

4. Recombinant vaccinia-EBNA1 stimulated bulk CTL cultures.

H. Chromium release assays.

1. Infection of target cells with vaccinia-EBV constructs.

2. Chromium release assays.

3. Inhibition of CTL-mediated killing by mAb.

4. Cold target competition assays.

I. Cytofluorographic analysis.

J. Reagents.

1. Recombinant vaccinia virus vectors.

2. Cell lines and corresponding MHC class-I haplotypes.

3. Monoclonal antibodies and antigen specificities.

4. Recombinant human lymphokines.

5. Immunobeads: rabbit α-human IgM coated beads.

6. Miscellaneous reagents.

CHAPTER III. RESULTS

A. EBV strain typing by DNA-PCR.

B. Expression of single EBV gene products in target cells.

1. Immunofluorescence detection of EBV gene products.

2. Immunoblot detection of EBV gene products.

C. Cytofluorographic analysis of EBV-primed CTL.

1. Activation marker expression on CD8+ T cells during acute IM.

2. Activation marker expression on in-vitro primed memory CTL.

D. EBV-specific HLA-restricted CTL from blood of EBV-seropositive donors.

1. EBV-specific CTL lyse cells expressing single EBV genes.
E. In-vivo primed CTL derived directly from the peripheral blood of acute IM patients display EBV-specificity and HLA-restriction in-vitro.

1. Target lysis by acute IM CTL is Ag-specific and HLA class-I restricted.
2. Acute IM CTL lyse target cells expressing single EBV genes.
3. In-vivo primed, EBV-specific CTL are CD8+/class-I restricted.
4. In-vitro primed memory CTL recognize predominantly EBNA3 antigens.

F. Recombinant vaccinia-EBNA1 stimulated CTL.

1. Vac-EBNA1 stimulated CTL from EBV sero+/vaccinia sero+ donors.
2. Vac-EBNA1 stimulated CTL from acute IM patients.
3. Identification of antigen source by cold target competition assay.

CHAPTER IV. DISCUSSION

1. Role of CTL immunity against EBV.
2. Primary vs Memory CTL responses.
3. Clinical significance of EBNA-1 expression in malignant cells.
4. Evidence against existence of EBNA-1-specific CTL.
5. Possible explanations for reported absence of EBNA-1-specific CTL.
6. EBNA-1-specific CTL activity is present during primary EBV infection.
7. Integration of EBNA-1-specific CTL into EBV immunobiology.
8. Implications for EBV vaccine strategies.
9. MHC class-I restriction elements for EBV derived antigens.
10. Phenotype of effector cells.
11. In-vitro growth of EBNA-1-specific CTL.

TABLES
FIGURES
REFERENCES
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.</td>
<td>Summary of Patient Information.</td>
<td>142</td>
</tr>
<tr>
<td>Table 2.</td>
<td>Established cell lines and corresponding MHC class-I haplotypes.</td>
<td>143</td>
</tr>
<tr>
<td>Table 3.</td>
<td>Specifications of recombinant vaccinia virus constructs.</td>
<td>145</td>
</tr>
<tr>
<td>Table 4.</td>
<td>Monoclonal antibodies &amp; corresponding antigen specificities.</td>
<td>146</td>
</tr>
<tr>
<td>Table 5.</td>
<td>Activation Marker Expression on CD8+ T Cells from IM Patients.</td>
<td>147</td>
</tr>
<tr>
<td>Table 6.</td>
<td>Summary of Primary vs Secondary EBV-Specific CTL Responses.</td>
<td>148</td>
</tr>
<tr>
<td>Table 7.</td>
<td>Acute IM patient CTL responses specific for EBNA-1.</td>
<td>149</td>
</tr>
<tr>
<td>Table 8.</td>
<td>Acute IM patient CTL responses specific for EBV-gp350.</td>
<td>150</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1. EBV strain typing by DNA-PCR amplification. 151
Figure 2. FACS analysis of SRBC-rosette separated lymphocytes. 152
Figure 3. Immunofluorescence detection of EBV nuclear proteins. 153
Figure 4. Immunofluorescence detection of EBV membrane proteins. 154
Figure 5. Immunoblot detection of EBNA1, 2, LP proteins. 155
Figure 6. Immunoblot detection of EBNA3A,B,C & LMP1 proteins. 156
Figure 7. Surface activation marker expression on CD8+ T cells during acute IM. 157
Figure 8. Kinetics of HLA-DR & CD11b expression on CD8+ T-cells during acute IM. 158
Figure 9. Kinetics of HLA-DR expression on CD8+ T-cells during acute IM. 159
Figure 10. Kinetics of HLA-DR & M01 expression on CD8+ T-cells during acute IM. 160
Figure 11. Kinetics of activation marker expression on CD8+ T-cells following acute IM. 161
Figure 12. In-vitro growth of EBV-specific CTL from blood of seropositive donors. 162
Figure 13. EBV-specific memory CTL lyse targets expressing single EBV genes. 163
Figure 14. Acute IM patient CTL lyse EBV-infected, HLA-compatible targets. 164
Figure 15. Composite of primary CTL responses to EBV infection. 165
Figure 16. Acute IM CTL mediated lysis is blocked by anti-class-I and anti-CD8 mAb. 166
Figure 17. Surface phenotype of in-vivo primed EBV-specific CTL. 167
Figure 18. Composite of in-vitro primed memory CTL responses to EBV. 168
Figure 19. Primary & secondary EBV-specific CTL responses in individual patients. 169
Figure 20. Unique memory CTL response to EBV which includes EBNA1 reactivity. 170
Figure 21. Graphical comparison of primary and memory CTL responses to EBV. 171
Figure 22. Target cell killing by CTL stimulated with Vac-EBNA1. 172
Figure 23. Target cell lysis by acute IM patient CTL expanded with Vac-EBNA1. 173
Figure 24. Identification of antigen source by cold target competition assay. 174
LIST OF FIGURES

(continued)

Figure 25. Surface marker analysis of Vac-EBNA1 stimulated bulk CTL culture. 175
Figure 26. TCR phenotype of Vac-EBNA1 stimulated bulk CTL culture. 176
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABS</td>
<td>Human AB serum</td>
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<tr>
<td>ADCC</td>
<td>Antibody dependent cell mediated cytotoxicity</td>
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<td>AET</td>
<td>2-Aminoethylisothiouronium bromide</td>
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<tr>
<td>BLCL</td>
<td>B lymphoblastoid cell line</td>
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<tr>
<td>2ME</td>
<td>2-mercaptoethanol</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
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<tr>
<td>EBNA</td>
<td>Epstein-Barr virus nuclear antigen</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>E:T</td>
<td>Effector to Target cell ratio</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>HBSS</td>
<td>Hanks buffered saline solution</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>HTLV-1</td>
<td>Human T lymphotrophic virus 1</td>
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<tr>
<td>HVS</td>
<td>Herpesvirus saimiri</td>
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<tr>
<td>IL-2</td>
<td>Interleukin 2</td>
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<tr>
<td>IL-4</td>
<td>Interleukin 4</td>
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<tr>
<td>LMP</td>
<td>Latent membrane protein</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<tr>
<td>NK</td>
<td>Natural killer cell</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>R:S</td>
<td>Responder to stimulator ratio</td>
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<tr>
<td>Vac</td>
<td>Vaccinia virus</td>
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<tr>
<td>VCA</td>
<td>Viral capsid antigen</td>
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<tr>
<td>XLP</td>
<td>X-linked lymphoproliferative syndrome</td>
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</tbody>
</table>
CHAPTER I
INTRODUCTION

History of Epstein-Barr Virus

**Discovery & Classification of EBV.** Diseases caused by virus infections have plagued man for centuries and represent some of the most formidable scourges of humans including such virulent illnesses as rabies, AIDS, poliomyelitis, small pox and yellow fever. While attempts by man to prevent disease through virus eradication have clearly succeeded as evidenced by the virtual elimination of small pox (Bloch, 1993), today, viral infections remain a forerunner as the cause of human ailments. Quite possibly the most intriguing aspect of viral illnesses involves the heterogeneity of disease manifestations caused not only by disparate viruses but also by a single strain of virus infecting different individuals. Variables such as genetic makeup, previous virus exposure, underlying illness, route of infection, age and so on, all contribute to the clinical picture and course of illness. Typically, diseases caused by viruses are first recognized by the presentation of patients with a characteristic set of signs and symptoms whose onset closely correlates with exposure to the suspected source of the infectious agent. While this scenario holds true for many acute viral infections such as smallpox and rabies (Baer, 1994), it differs markedly from the insidious onset of illness which follows exposure to HIV. Accordingly, the search for viruses which cause human disease is frequently plagued with inconsistencies in clinical presentation and exposure history.

In the century preceding the discovery of EBV, various clinicians had speculated on an infectious etiology to explain a common clinical syndrome initially termed “glandular fever” (Pfeiffer and Kinderh, 1889), characterized by fever, tonsilar adenopathy, splenomegaly and mononuclear leukocytosis (Sprunt and Evans, 1920). Three years later Downey described the now classic atypical lymphocytes which provided a more accurate marker for recognizing glandular fever, later renamed “infectious mononucleosis” (IM) (Downey and McKinlay, 1923).
Almost a decade later, Paul and Brunnell described high titers of spontaneously occurring heterophile antibodies in the sera of patients with infectious mononucleosis (Paul and Bunnell, 1932). Initial attempts to isolate the etiologic agent were unsuccessful as were attempts to transmit the disease to humans (Evans, 1950). A systematic analysis to identify the etiologic agent did not occur until the late 1950's when British surgeon Dennis Burkitt suggested a causal relationship between a common tumor of children in equatorial Africa and an infectious agent (Burkitt, 1958)(Burkitt, 1962). This idea immediately attracted the attention of an English pathologist named Anthony Epstein who began searching for an oncogenic virus in Burkitt's lymphoma cells grown in vitro (Epstein and Barr, 1964). In 1964 Epstein's lab analyzed tumor biopsies by thin-section electron microscopy and discovered a new, large, icosahedral herpesvirus which could be directly reactivated from in vitro grown Burkitt lymphoma cells. The initial findings were reported in Lancet and the virus was later named after Epstein and his graduate student Yvonne Barr (Epstein et al., 1964). Shortly thereafter, two independent groups (Henle et al., 1967)(Pope et al., 1968) reported on the ability of EBV to transform primary human B lymphocytes into permanently growing lymphoblastoid cell lines, providing the first concrete evidence to support a causal role for EBV in the establishment of a human cancer. It was during this same period of time that a technician in the Henle laboratory whose blood was being used as a negative control in serum antibody tests contracted infectious mononucleosis and simultaneously developed a high titer of EBV specific antibodies (Henle et al., 1968), thus providing the first convincing evidence that EBV was the cause of infectious mononucleosis-glandular fever. Several large-scale sero-epidemiologic prospective studies subsequently showed conclusively that EBV was the causative agent of infectious mononucleosis (Evans et al., 1968) (Niederman et al., 1968). Exposure to the virus is exceedingly common, as sera from 90% of American adults contain EBV-specific antibodies, indicating a high prevalence of subclinical infections.

Further evidence for the causal role of EBV in human cancers came from early studies
involving non-human primates, which when infected with EBV rapidly developed malignant lymphomas (Shope et al., 1973). Fortuitously, these cotton top tamarin lymphomas such as the B95-8 line grow readily in vitro, and unlike most human cell lines, are permissive for virus replication (Miller et al., 1972) thus providing a continued source of EBV for research purposes.

EBV is a member of the gamma herpesvirus family and is the prototype for the lymphocryptovirus genus (Roizman, 1993). In vitro, all gamma herpesviruses replicate in lymphoid cells and some are capable of lytic replication in epithelial and fibroblastic cells. The host range of the lymphocryptovirus genera is restricted to primate B lymphocytes which are also the site of latent virus infection in vivo. Infection of primate B lymphocytes with lymphocryptoviruses typically results in a latent infection characterized by persistence of the viral genome along with expression of a restricted set of latent gene products which contribute to the transformation process and help drive cell proliferation (Kieff and Liebowitz, 1990).

**Virus & Genome Structure.** Herpesviruses are highly disseminated among most animal species and nearly 100 have been partially characterized, eight of which have been isolated from humans [HSV-1, HSV-2, HCMV, VZV, EBV, HHV6, HHV7, and HHV8] (Roizman, 1993). The most recently characterized, HHV8, is associated with kaposi's sarcoma and abdominal lymphomas. Membership in the Herpesviridae family is based upon virion architecture. EBV consists of a toroid-shaped protein core wrapped with linear double-stranded DNA, an icosahedral nucleocapsid containing 162 capsomeres, an amorphous protein tegument surrounding the capsid, and an outer envelope containing glycoprotein spikes (Liebowitz and Kieff, 1993).

Similar to HSV-1, the major EBV capsid proteins range in size from 28-160 KDa (Dolyniuk et al., 1976) , however, unlike most other herpesviruses, the outer viral envelope of EBV consists of predominantly a single glycoprotein known as gp350/220 (Dolyniuk et al., 1976)(Thorley-Lawson and Edson, 1979).
The EBV genome was first characterized in 1970 and consists of a linear, 172-kilobase-pair, double-stranded DNA molecule composed of 60 mole % guanine and cytosine (Pritchett et al., 1975). In the late 1970's, DNA from the entire B95-8 EBV genome was cloned into bacterial plasmids (Dambaugh et al., 1980), and was the first herpesvirus genome to be completely sequenced (Baer et al., 1984). The characteristic features of the EBV genome include a single overall format and gene arrangement, tandemly reiterated 0.5 Kbp terminal direct repeats (TR), and tandemly reiterated 3 Kbp internal direct repeats (IR) which divide the genome into long and short, predominantly unique regions (UL and US). These characteristic DNA repeat elements (IR) serve as important, unique landmarks on the EBV genome which allow one to distinguish between EBV strains (Bornkamm et al., 1980). While various EBV isolates differ in their tandem repeat frequency, individual EBV isolates tend to contain a constant number of internal repeats even through virus replication and serial passage to new B cells (Dambaugh et al., 1980). In contrast, the terminal repeats are covalently linked during episome formation, and only remain constant during latent replication from ori-p. During lytic replication the terminal repeats are randomly cleaved and thus do not remain constant during serial passage. This principle is extremely useful in determining whether or not latently infected cells, such as Burkitt's lymphoma, arise from a single progenitor (monoclonal) or multiple progenitor cells, as in polyclonal posttransplant lymphomas (Brown et al., 1988). The following is a schematic depiction of the linear EBV genome showing the location of EBV genes expressed during viral latency.
Each 3Kbp direct IR contains a Wp promoter, which is the dominant promoter in latent infection. Origin of replication in latent infection.

Overall, there is general conservation of the genetic organization between herpesvirus saimiri (HVS) and Epstein-Barr virus, although the homology to HVS is disrupted by unique EBV DNA segments which function in latent B cell infection (Nicholas et al., 1992). Antigenic cross-reactivity between EBV and other herpesviruses is rare, even among the proteins encoded by the more conserved genes. In fact, the EBV genes expressed in latent infection as well as several lytic cycle genes have no detectable homology to other herpesvirus genes and many believe they may have arisen in part from cellular DNA (Heller et al., 1982) (Heller et al., 1985). In particular, an irregular repeat motif GGGGCAGGA present in the latent cycle EBV nuclear antigen 1 (EBNA1) is also interspersed in human cell DNA (Heller et al.,...
Other examples of EBV lytic cycle genes with significant homology to the human genome but little homology to other herperviruses include BZLF1, BHRF1, and BCRF1. BZLF1 is an immediate early gene closely related to the fos and jun transcriptional activators (Packham et al., 1990). BHRF1 is an EBV early gene with significant homology to the human bcl-2 gene (Cleary et al., 1986) thought to be involved in preventing B cells and other cells from undergoing apoptosis. Lastly, BCRF1 is an EBV late gene with nearly identical primary amino acid sequence homology and biological activity to human IL-10 (Hsu et al., 1990).

Type & Strain Variations. Two types of EBV, formerly designated EBV type-A and -B, now referred to as EBV-1 and EBV-2 so as to parallel the HSV-1 and HSV-2 nomenclature (Liebowitz and Kieff, 1993) have been identified in most human populations (Bornkamm et al., 1980)(Dambaugh et al., 1980)(Sample et al., 1990)(Zimber et al., 1986). Unlike HSV-1 and HSV-2, however, there is extensive homology and restriction endonuclease site conservation throughout most of the EBV-1 and EBV-2 genomes (Dambaugh et al., 1984). The major identified differences between the EBV-1 and EBV-2 genomes exist in the latent infection cycle nuclear antigen genes EBNA-2, EBNA-LP (Dambaugh et al., 1984), EBNA-3A, -3B, and -3C (Rowe et al., 1989) and in the small, non-polyadenylated RNAs EBERs 1 & 2 (Arrand et al., 1989). As expected, the differences between EBV-1 and EBV-2 EBNA genes are reflected in type-specific and type-common EBNA epitopes (Hennessy and Kieff, 1985). Similarly, the immune recognition of BLCLs by EBV type-specific CTL is dependent upon the infecting EBV strain (Moss et al., 1988)(Misko et al., 1991)(Moss et al., 1992). The prevalence and geographic distribution of the two EBV strains has been determined by serologic reactivities to EBNA-2. Results indicate that African-derived EBV genomes are almost as frequently type-2 as type-1 (1:2), which contrasts with American and European EBV genomes which are 10 times more likely to be type-1 than type-2. Similar serologic findings extend to the EBNA-3A, -3B, and -
3C genes (Rowe et al., 1989). Sera from type-1 EBV-infected patients preferentially react with type-1 EBNA-3A, -3B, and -3C, whereas sera from individuals infected with type-2 EBV react preferentially with type-2 EBNA-3 gene products. With respect to homology, the EBNA gene products share between 50-85% primary amino acid sequence identity, specifically, the type-1 EBNA-2, -3A, -3B, and -3C differ in predicted primary amino acid sequence from their type-2 counterparts by 47%, 16%, 20% and 28% respectively (Sample et al., 1990). There also exists limited genomic divergence between various type-1 EBV isolates. In one study 2% nucleotide sequence and 5% amino acid sequence divergence was observed between two EBV-1 latent membrane protein-1 (LMP-1) genes (Hatfull et al., 1988). Importantly, the EBV type associated with various malignancies appears to correlate well with the degree of immune dysfunction. For example, type-1 EBV is present in virtually all EBV-associated Hodgkin’s disease tumors (Gledhill et al., 1991), except those in AIDS patients (Boyle et al., 1991), in essentially all nasopharyngeal carcinomas (Chen et al., 1992), and two thirds of African Burkitt’s lymphomas. In contrast, type-2 EBV is present in approximately one half of EBV-associated lymphomas in AIDS patients, and virtually all EBV-associated T cell lymphomas (Borisch et al., 1993).

**Host Range & Virus Receptor.** The host range of Epstein-Barr virus is restricted to humans and certain sub-human primates including squirrel monkeys and cotton top marmosets (Miller et al., 1972). In general, the host range of lymphocryptoviruses is restricted to immature B lymphocytes which selectively express a cell surface protein which also serves as a virus receptor. In vitro, B cell infectivity with EBV correlates with the immature stages of development, when the highest density of a 140 kDa glycoprotein (CR2, now renamed CD21) receptor is present, making it a likely candidate for the virus receptor (Jondal et al., 1976)(Aman et al., 1984) (Fingeroth et al., 1984) (Frade et al., 1985). The identity of the EBV receptor was confirmed in several studies showing that 1) purified CD21 binds to EBV
(Nemerow et al., 1986), 2) virus infection is blocked by antibody directed against the CD21 glycoprotein (Nemerow et al., 1987), and 3) expression of CD21 on heterologous cells confers binding to EBV (Ahearn et al., 1988). CD21 is also the receptor for the C3d component of complement. For review see (Cooper et al., 1988).

The initial stage of infection involves the interaction of the major EBV outer envelope glycoprotein gp350/220, with the B cell surface molecule CD21 (Tanner et al., 1987). Currently, gp350 is believed to bind exclusively to the CD21 molecule as it does not bind to any other B cell surface molecules. Comparison of the primary amino acid sequences of gp350 and C3d has revealed a shared nonapeptide (EDPGFFNVE) which likely explains their common binding properties with CD21 (Lambris et al., 1985).

In humans, the majority of primary EBV infections are believed to originate in oropharyngeal lymphocytes (Anagnostopoulos et al., 1995), and oropharyngeal epithelial cells (Sixbey et al., 1983)(Li et al., 1992). In fact, double-label in-situ hybridization studies performed by Anagnostopoulos suggest that EBV infection is restricted to B- and T-lymphoid cells, as they found no evidence of EBV infected oropharyngeal epithelial cells in 9 of 9 acute IM patients examined. Accordingly, while the identity of the virus receptor on B cells is clearly the type-2 complement receptor (CR2/CD21) (Fingeroth et al., 1984), the EBV receptor on epithelial cells, if one exists, remains unknown. In general, EBV binds much less efficiently to epithelial cells than B cells, and most anti-CD21 antibodies do not bind to epithelial cells. These findings have led some to suggest the presence of a 200 kDa CD21 homologue on normal epithelial cells (Sixbey et al., 1987). More recently, northern blot hybridization has been used to demonstrate the presence of small amounts of CD21 mRNA which is indistinguishable in size from that expressed in B cells. Additionally, cloning and sequencing of the epithelial cell derived RNA has shown it to be identical to the B cell derived CD21 (Birchenbach et al., 1991).

A large body of evidence suggests the presence of CD21, or related structures, are also
present on cells of the T lineage (Tsoukas and Lambris, 1993), and T lymphocytes are known to be EBV-infected during acute IM (Anagnostopoulos et al., 1995). Both thymocytes and peripheral T cells express CD21 or CD21-like molecules, however, their reactivity with anti-CD21 antibodies differs from that of B cells, suggesting there may be structural differences between T- and B-cell CD21 molecules. Low levels of the CD21 receptor are also expressed on established T cell lines such as MOLT-4, and T cell lines (MT-2) transformed with the human T cell leukemia virus 1 (HTLV1) (Koizumi et al., 1992).

Stages of Infection. In general, the herpesviruses undergo lytic replication in one cell type while simultaneously establishing latent infection in another, indicating that cell specific factors determine the overall balance of virus/cell interactions. In keeping with the replication of human herpesviruses in epithelial cells, large amounts of EBV have been reported to be present in squamous epithelial cells recovered from the oropharynx of persons with acute infectious mononucleosis (Lemon et al., 1977). In fact, the oropharyngeal epithelium is believed to be permissive for viral replication and was once considered to be the site of primary EBV infection (Sixbey et al., 1984). Recent data convincingly demonstrate that lymphocytes, and not epithelial cells, are the primary cells infected during acute IM (Anagnostopoulos et al., 1995). These EBV infected lymphocytes are present in the crypt epithelium of palatine tonsils and are the likely means of EBV transfer to uninfected individuals. During primary EBV infection, persistent active replication ensues which then continues at some level for many years, usually accompanied by prolonged oropharyngeal virus excretion (Miller et al., 1973). Early in the course of primary infection, EBV infects small resting B lymphocytes which traffic through the oropharynx close to the epithelial basement membrane. The virus rarely undergoes lytic replication in B cells, but instead establishes a latent type of infection whereby the genome is maintained as an episome and gene expression is restricted to the EBNAs, LMPs and EBERs, thereby disseminating the viral genome through
cellular proliferation. The same process can also occur in reverse, as shown in studies involving the analysis of EBV strains in donor recipient pairs, prior to, and following bone marrow transplantation. In one case, a bone marrow recipient’s strain of EBV disappeared from the oropharynx following radiation induced immune ablation and was subsequently replaced with the EBV strain of the marrow donor (Gratama et al., 1988)(Gratama et al., 1990). This finding also lends credence to the theory that B cells, and not epithelial cells, are the reservoir for EBV persistence (Miyashita et al., 1995). The following diagram depicts the pathway leading from primary oropharyngeal infection with EBV through the establishment of latency in B cells. In this model, cell-mediated immunity (CD8+ CTL) limits the proliferation of latency type-III EBV-infected B cells (immunoblastic phenotype) in the peripheral blood, which only rarely enter lytic cycle replication and give rise to progeny virions. EBV-infected cells with a latency type-I phenotype (EBNA-1 expression in quiescent B cells) serve as an immunologically protected reservoir for EBV persistence.
**Model for Primary EBV Infection and Persistence**

1) **Primary Infection**

- EBV adsorption, penetration, uncoating: EBV infection is initiated by interaction of the virus with the CD21 molecule on epithelial or B cells. Virus binding as well as the initial phase of penetration are mediated through the major viral coat glycoprotein gp350/220 (Wells et al., 1982) (Tanner et al., 1987). As mentioned earlier, the only B-lymphocyte surface molecule known to bind gp350/220 is CD21 (Nemerow et al., 1987) and EBV infection can be blocked by monoclonal antibody directed to either CD21 or gp350 (Hoffman et al., 1980). Further evidence supporting the essential role of gp350/CD21 interaction during EBV

2) **Latency**

- EBNA1
- EBNA1, 2
- 3A, 3B, 3CLP
- EBER1 & 2
- LMP1, 2A, 2B
- EBV Reservoir
- EBV Repository
- EBV Ags

3) **Viral Persistence**

- Palatine Tonsil
- Crypt Epithelium
- NPC

- Switch from viral latency to lytic replication and virus shedding

Adapted from Liebowitz and Kieff, in: The Human Herpesviruses (1993)
adsorption is the demonstration that soluble gp350 can block virus infection by saturation of available CD21 binding sites (Tanner et al., 1988). Virus adsorption on the surface of B cell results in capping of CD21, followed by endocytosis of EBV into smooth membrane vesicles (Nemerow and Cooper, 1984). A second EBV envelope glycoprotein (gp85) then mediates fusion of the virus with the vesicle membrane, causing release of the nucleocapsid into the B cell cytoplasm (Miller and Hutt-Fletcher, 1988).

Dissolution of the viral nucleocapsid and transport of the genome to the cell nucleus are less well understood. Once inside the nucleus, the linear EBV genome circularizes (Adams and Lindahl, 1975) (Lindahl et al., 1976), which precedes, or at least coincides with the earliest gene expression (Alfieri et al., 1991), directed from the first latent infection promoter Wp. The EBV genome is replicated by cellular DNA polymerases during the cell cycle S phase (Adams, 1987), and persist as multiple, extrachromosomal double stranded EBV episomes, which are organized into nucleosomes similar to chromosomal DNA (Dyson and Farrell, 1985).

Virus Expression in Latent Infection. The hallmark of B lymphocyte infection with EBV is the establishment of latency, which is characterized by three distinct processes, including (i) viral persistence, (ii) restricted virus expression which alters cell growth and proliferation, and (iii) retained potential for reactivation to lytic replication. Intracellular persistence of the entire viral genome is achieved through circularization of the linear EBV genome present in viral particles, and maintenance of multiple copies of this covalently closed episomal DNA (Adams, 1987). The episomes are replicated semi-conservatively during cell cycle S-phase by cellular DNA polymerases, and equal partitioning of episomes to daughter cells is mediated by interactions between the latent origin of plasmid replication (OriP) and EBV nuclear protein 1 (EBNA1) (Yates et al., 1984). The 172 Kbp EBV genome encodes approximately 100 genes, ten of which are expressed during latency and are thought to be involved in establishing and maintaining the "immortalized" state, including six EBV nuclear
proteins (EBNAs 1, 2, 3A, 3B, 3C, LP), two latent membrane proteins (LMP-1 and 2), and two small untranslated, non-polyadenylated RNAs (EBERs 1, 2) (Liebowitz and Kieff, 1993). Latency can be disrupted through a variety of cellular activators, resulting in expression of BZLF1, a key mediator which induces the switch from viral latency to lytic replication (Flemington and Speck, 1990) (Packham et al., 1990).

Unlike the short term proliferation induced by crosslinking surface Ig on B cells, proliferation of B lymphocytes driven by EBV transformation is indefinite. In spite of this difference in proliferation potential, the phenotype of antigen driven B lymphocytes is similar to that induced by EBV, which transforms B cells through autocrine growth mechanisms that promote or at least maintain a differentiated B cell phenotype. This is in marked contrast to the poorly differentiated phenotype characteristic of most Burkitt lymphomas carrying the c-myc translocation, many of which express the germinal center B cell marker CD10 but do not express activation (CD21, CD23, CD80) or adhesion molecules (LFA-1, LFA-3, ICAM-1).

While only about 10% of the genes encoded by EBV are expressed in latently infected B cells, the transcribed regions encompass a major portion of the viral genome. Transcription of the EBNA and LMP genes is mediated by cell derived RNA polymerase II, while the EBERs are primarily transcribed by RNA polymerase III (Arrand and Rymo, 1982) (Howe and Shu, 1989). By far, the most abundantly transcribed EBV genes in latently infected cells are the EBERs (10^7 copies/cell), distantly followed by LMP1, which in turn is significantly more abundant than the EBNAs and LMP2. The majority of the genome 3' of the Wp promoter is transcribed, however, the selection of specific promoters and alternate splicing ultimately determine the levels of latent gene expression (Sample and Kieff, 1990). The following diagram depicts the extensive transcription and long-range splicing of the EBV genome which occurs in latently infected B lymphocytes.
Transcription of EBV Episome in Latently Infected B Lymphocytes

Following circularization of the linear EBV genome within the cell nucleus, rightward transcription of the EBNAs is initiated from the Wp promoter within IR1, a copy of which is encoded in each 3kb long internal repeat (Sample et al., 1986). The EBNA mRNAs are assembled by alternative splicing and 3' processing of a common precursor encoded by over 100 kb of the genome (Bodescot and Perricaudet, 1986) (Sample and Kieff, 1990). EBNA LP and EBNA 2 are the first EBV proteins expressed during latent infection of B cells, and reach their steady state levels within 24-32 hrs (Alfieri et al., 1991). EBNA 2 is essential to the immortalization process as viruses with deletions encompassing EBNA 2, such as P3J-HR-1 and Daudi, are immortalization incompetent (Bornkamm et al., 1982) (Jones et al., 1984). Infection
of primary B cells with the EBNA 2 mutant HR-1, not only results in failure of EBNA 2 expression, but also in failure to express EBNA genes not deleted in HR-1 such as EBNA 1 and EBNA 3 genes (Rooney et al., 1989). Thus, EBNA 2 is required for expression of other EBV latent genes, and has been shown to transactivate cellular genes including CD23 (Wang et al., 1987), CD21 (Wang et al., 1990), c-fgr (Knutson, 1990), and EBV genes including LMP 1 and LMP 2 (Abbot et al., 1990)(Rooney et al., 1992).

EBNA 2 also likely plays a role in promoter switching during the initial stages of latent B cell infection. Promoter usage in some cells has been shown to move upstream from the dominant latency promoter Wp to an alternative latency promoter Cp (Woisetschlaeger et al., 1990)(Woisetschlaeger et al., 1991). All resulting Wp or Cp initiated transcripts have the same repeating exons (W1 and W2) in their leader sequences. A potential splice donor site near the 5' end of the EBNA 2 exon which preceeds the EBNA 2 initiation codon is activated in some transcripts, resulting in alternate splicing to a far downstream acceptor in U3. The U3 exon is then alternately spliced to any one of four acceptor sites in the ORFs that encode EBNA3A, 3B, 3C and 1 (Bodescot and Perricaudet, 1987). LMP1 and LMP 2 are transcribed in opposite directions under the control of closely positioned promoters sharing the same EBNA2 response element. By 32 hr post-infection, all of the EBNA proteins and LMP1 can be detected using appropriate antisera (Alfieri et al., 1991). While less is known about onset of expression of LMP 2A and LMP 2B due to lack of good quality antisera, their regulation appears to be similar to that of the other EBNA proteins, as both of the LMP 2 promoters are EBNA2 responsive.

Concomitant with LMP1 expression is a further increase in the level of CD23 and the onset of cell DNA synthesis. Expression of the EBNA proteins reaches a steady state level within 48 hours of primary B cell infection (Allday et al., 1989) (Moss et al., 1981).

In contrast to EBV transformed lymphocytes grown in vitro, many EBV-carrying tumors grown in vivo, do not express the full spectrum of latent proteins (Rowe et al., 1986) (Rowe et al., 1987), unless they are derived from an immunocompromised host (Young et al., 1989). In the
case of endemic Burkitt’s lymphoma (BL), most latent cycle genes are not expressed, while EBNA-1 is expressed from an alternative promoter, Fp or more correctly Qp (Schafer, 1995 J. Virol.), located downstream of the EBNA2 gene (Sample et al., 1991). Presumably, the Cp and Wp promoters are suppressed in BL, and the Qp promoter is inactive in B-LCLs. This specific downregulation of other latent proteins demonstrates the significance of tissue-specific regulation of latent genes in vivo, as EBNA1 does not induce adhesion molecules required for cytotoxic T-cell (CTL) mediated killing (Rowe et al., 1986), thus contributing to the lack of recognition of BL cells by class-I-restricted CTLs (Rooney et al., 1985).

Transformation and Latent Proteins. All latently infected B cells harbor one, or usually more, complete EBV episomes and when grown in vitro express a restricted set of latent genes comprised of six nuclear antigens (EBNA 1, 2, 3A, 3B, 3C and LP), two latent membrane proteins (LMP 1 and 2) and two untranslated, nonpolyadenylated RNAs (EBER-1 and EBER-2). While all of the EBNA genes are expressed in latently infected B lymphocytes transformed in vitro, considerable variation in EBNA gene expression exists among various EBV-infected cells growing in-vivo (Miyashita et al., 1995) (Gregory et al., 1990) (Rowe et al., 1987). The family of EBV-encoded nuclear antigens (EBNA) was originally named as such based upon the ability of antisera from persons previously infected with EBV to recognize these proteins in the nuclei of infected cells.

S1 nuclease protection and DNA sequencing were used to identify the first EBNA protein (EBNA1) which encodes an irregular glycine-alanine repeat whose length varies according to the strain of EBV (Hennessy et al., 1983). Based upon the predicted primary amino acid sequence, EBNA 1 consists of 641 amino acids, is highly charged and has an apparent M.W. of 76 KDa (Hearing et al., 1984). While most of the EBNA proteins bind DNA cellulose and are therefore presumed to have DNA binding capability, only EBNA-1 has sequence-specific DNA binding properties, and binds to three cognate sites (TAGGATAGCATATGCTACCCAGATCCAG)
located on the EBV genome (Rawlins et al., 1985) (Jones et al., 1989). The EBNA1 cognate sequence having highest binding affinity consists of 20 tandem 30 bp repeats (region 1), while the site having the second highest affinity consists of two cognate sequences in dyad symmetry and two in tandem (region 2). Together, the site of dyad symmetry and the 20 tandem direct 30 bp repeats constitute the origin of plasmid replication (OriP) (Reisman et al., 1985), which is required for efficient episome persistence and replication in latently infected cells (Wysokenski and Yates, 1989) (Lupton and Levine, 1985) (Yates et al., 1984). Region 2 contains four EBNA1 binding sites, is located near the initiation site of latent cycle DNA replication, and is absolutely required for episome replication and maintenance (Gahn and Schildkraut, 1989). Likewise, region 1 is absolutely required for the maintenance of plasmids containing region 2 in cells expressing EBNA1, and region 1 contains the termination site for replication. Lastly, during metaphase, EBNA1 is diffusely associated with all chromosomes in latently infected cell nuclei, and it is through this combined interaction of EBNA1 with both OriP and chromosomal proteins which assures equal partitioning of EBV episomes to progeny cells (Harris et al., 1985). EBNA-1 also transactivates the EBNA-2 protein, which in turn, activates production of two latent membrane proteins (LMP-1 and -2), as well as several B-cell encoded products CD21, CD23 and c-fgr.

EBNA 2 is essential to the process of B lymphocyte immortalization as viruses having deletions encompassing EBNA 2, such as P3-HR-1 and Daudi, are unable to transform primary B lymphocytes into permanently growing B-lymphoblastoid cell lines (B-LCLs) (Miller et al., 1974) (Bornkamm et al., 1982) (Jones et al., 1984). Infection of primary B cells with the EBNA 2 mutant P3HR-1, not only results in failure of EBNA 2 expression, but also in failure to express EBNA genes not deleted in P3HR-1 such as EBNA 1 and EBNA 3 genes (Rooney et al., 1989). Restoration of the deleted DNA in defective P3HR-1 EBV produces progeny virus with the ability to transform primary human B lymphocytes (Skare et al., 1985) (Cohen et al., 1989).

Two major types of EBV exist, types 1 and 2, and variations in the EBNA2 protein
impart the most significant biologic difference between these two EBV types. In general, type 1 EBV (B95-8) transforms normal human B lymphocytes much more efficiently than type 2 EBV (P3J-HR-1 and its nondeleted parental strain Jijoye) (Rickinson et al., 1987). Confirmation of the critical role played by EBNA2 in the transformation process was made by inserting cloned type 1 EBNA2 DNA into the P3HR-1 deletion site, which conveyed to it a highly efficient transformation phenotype identical to that of type 1 EBV (Cohen et al., 1989).

The predicted primary amino acid sequence of type 1 EBNA2 consists of 491 amino acids (90 KDa), while that of type 2 EBNA2 consists of 443 amino acids (78 KDa) (Hennessy and Kieff, 1985) (Mueller-Lantzsch et al., 1985). Overall, EBNA2 is an acidic molecule with several domains, one of which, located in the carboxy terminus, is responsible for nuclear localization (Cohen et al., 1991).

The first biochemical evidence for a role of EBNA2 in B cell growth-transformation came from the demonstration that EBNA2 specifically transactivates expression of the B lymphocyte activation marker CD23 (Wang et al., 1987), which is abundantly expressed in EBV-transformed and antigen-primed B lymphocytes (Hurley and Thorley-Lawson, 1989). EBNA2 also upregulates expression of the cellular genes CD21 (Wang et al., 1990) and c-fgr (Knutson, 1990), and the EBV latent genes LMP1 (Abbot et al., 1990) and LMP2 (Zimber-Strobl et al., 1991). Thus, most of EBNA 2's role in B lymphocyte transformation comes from it's ability to transactivate cellular and EBV genes.

EBNA 3 consists of a family of three high molecular weight gene products (EBNA 3A, 3B, 3C) ranging in size from 145-165 kDa (Hennessy et al., 1985) (Hennessy et al., 1986) (Petti and Kieff, 1988) (Petti et al., 1988) (Sample et al., 1990). The EBNA 3 genes are located in tandem on the EBV genome and their mRNAs are the least abundant, with only several copies of each being present in latently infected cells (Kallin et al., 1986). Despite the low levels of EBNA 3 mRNA in latently infected cells, all of the EBNA 3 proteins accumulate in large intranuclear clumps which almost completely fill the nucleus, sparing only the nucleolus (Petti
et al., 1990). Much like EBNA 2, the EBNA 3 genes are polymorphic and differ according to the EBV type, 1 or 2. The sizes of the type 1 EBNA3s based upon predicted primary amino acid sequence are 3A=944, 3B=938, 3C=992 and share 84%, 80% and 72% amino acid identity, respectively, with their corresponding type 2 proteins (Rowe et al., 1989) (Sample et al., 1990). Unlike the difference in transformation phenotype imparted by the EBNA 2 type (1 or 2), the type specificity of the EBNA 3 genes (1 or 2) does not affect the ability of the virus to initiate growth transformation, episome maintainence or lytic replication (Tomkinson and Kieff, 1992). Systematic analysis of the transformation capability of EBV recombinants having specific mutations in each of the EBNA 3 genes demonstrated that while EBNA 3B is dispensable for B-lymphocyte growth transformation, mutations in either EBNA 3A or 3C renders the virus transformation incompetent (Tomkinson et al., 1993).

EBNA LP or leader protein is actually a set of highly polymorphic proteins encoded primarily by repeating exons (W1 and W2) from IR1 within the leader of the EBNA mRNAs. Translation is dependent upon a splice between exons downstream of either the Wp or Cp promoters and the first W1 exon. As the number of IR1 repeats varies among different EBV isolates, so does the size of EBNA LP, ranging from 22-70 kDa (Dillner et al., 1986). EBNA LP has a somewhat unusual nuclear location, being partially spread diffusely through the nucleus and also concentrated in several small nuclear granules. While the function of EBNA LP remains unclear, some have speculated that it may play a role in RNA processing or associate with some nuclear regulatory protein (Jiang et al., 1991), alternatively, it may function by upregulating expression of autocrine factors critical to B-LCL growth (Mannick et al., 1991). Strictly speaking, EBNA-LP is not required for transformation but it appears to augment growth transformation of primary B cells.

The second most abundant EBV mRNA species present in latently infected B lymphocytes (60 copies/cell) is highly stable and encodes a 63 kDa integral membrane protein LMP-1 (Fennewald et al., 1984) (Hennessy et al., 1985). While the LMP-1 promoter contains an
EBNA2 response element which functions to upregulate LMP-1 expression (Abbot et al., 1990), LMP-1 is expressed in the absence of EBNA2 during lytic cycle activation in BL cells (Contreras-Salazar et al., 1990), and in NPC tumors (Brooks et al., 1992).

Based upon predicted primary amino acid sequence, LMP-1 was correctly assumed to represent an integral membrane protein composed of a hydrophilic 20 amino acid cytoplasmic amino terminus, six highly hydrophobic, 20-amino-acid, transmembrane segments, and a highly acidic 180 amino acid cytoplasmic carboxy terminus. The plasma membrane location of LMP-1 was determined by measurement of LMP-1 content in various membrane fractions as detected with a mAb (S12) raised against a LMP-β-galactosidase fusion protein (Mann et al., 1985). Additionally, protease digestion of live cells and immunofluorescence revealed that 30% of LMP is located in the plasma membrane, and with the exception of a small outer reverse turn loop, the vast majority of LMP-1 is not exposed on the outer surface of the cell (Liebowitz et al., 1986).

In vitro, LMP-1 is essential for EBV-induced transformation of B cells into BLCLs (Kaye et al., 1993) (Kaye et al., 1995), and induces many of the activation markers associated with EBV infection of B lymphocytes (Rowe et al., 1994). Shortly after being translated, LMP-1 is phosphorylated and becomes tightly bound to vimentin, in association with the cytoskeleton (Liebowitz et al., 1987). The majority of LMP-1 is associated into discrete patches within the plasma membrane, which are often further assembled into a single caplike structure, a behavior characteristic of many activated receptors (Schechter et al., 1979). The process of LMP-1 cap formation does not require any additional EBV proteins, as LMP-1 continues to form patches when introduced by single gene transfer into the EBV-negative B-lymphoma cell line (Loukes) (Wang et al., 1988). This characteristic plasma membrane patching of LMP-1 prompted an exploration of its role in B-lymphocyte growth transformation. Transfer of the LMP-1 gene into continuous rodent fibroblast lines (NIH-3T3 and Rat-1), demonstrated multiple transforming effects, including altered cell morphology and reduced serum requirements (Wang
et al., 1985). In Rat-1 cells, LMP-1 expression induces a loss of contact inhibition and anchorage-independent growth in soft agar. Similar effects on cell growth by LMP-1 occur in NIH-3T3 cells (Wang et al., 1988). Importantly, Rat-1 cells, which are not normally tumorigenic in nude mice, become uniformly tumorigenic when expressing LMP-1 (Wang et al., 1985). The cytoskeletal association of LMP-1 appears to be integral to its activity, as a partially deleted form of the molecule (D1LMP) localizes to cytoplasmic membranes, does not form aggregates associated with the cytoskeleton and has little effect on B lymphocyte growth (Wang et al., 1988) (Moorthy and Thorley-Lawson, 1993). In many respects, the effects of LMP-1 on B lymphocyte growth resemble those mediated by activation of the CD40 receptor in the presence of IL-4 (Banchereau et al., 1994).

Expression of LMP-1 in EBV-negative Burkitt’s lymphoma lines induces many of the changes typically associated with EBV infection or antigen activation of primary B lymphocytes (Wang et al., 1990) (Wang et al., 1988) (Liebowitz et al., 1992). LMP-expressing cells exhibit increased villous projections and grow in tight clumps due to increased expression of the homotypic cellular adhesion molecules LFA-1 and ICAM-1 (Wang et al., 1988). This effect is advantageous to the virus as in-vitro grown BLCLs proliferate in discrete cell clumps which serve to enhance the effective concentration of secreted autocrine growth factors. Importantly, LMP-1 induction of the adhesion molecules LFA-1, LFA-3 and ICAM-1 promotes interaction between B and T lymphocytes via the LFA-3/CD-2 and LFA-1/ICAM-1 adhesion pathways. These heterotypic adhesions are important as the in-vivo elimination of EBV-transformed B lymphocytes is dependent on conjugate formation with cytotoxic T cells. Indeed, the levels of LFA-3 and ICAM-1 on infected cells influences their susceptibility to CTL lysis by modifying the affinity of effector-target conjugate formation (Makgoba et al., 1988). LMP-1 also induces cell surface expression of a number of B-cell activation molecules, including CD23, CD39, CD40, CD44 (Wang et al., 1990). More recently, LMP-1 has been shown to protect EBV infected B cells from programmed cell death (apoptosis), mediated in part through induction of
the cellular oncogene bcl-2 (Gregory et al., 1991) (Henderson et al., 1991).

LMP-2 is an integral membrane protein containing twelve hydrophobic transmembrane domains which co-localizes with LMP-1 in the plasma membrane of EBV infected lymphocytes (Longnecker and Kieff, 1990). Among the transformation associated EBV proteins, EBNA1, LMP-1 and LMP-2 are present most consistently in NPC tumor biopsies and EBV related malignancies (Young et al., 1989) (Brooks et al., 1992) (Busson et al., 1992). Since both LMP-1 and LMP-2 contain T cell epitopes (Murray et al., 1992), their persistent expression in-vivo argues for an important role in the persistence of EBV in the human host. The LMP-2 gene is simultaneously transcribed from two promoters, resulting in transcripts (LMP-2A and 2B) which are spliced into nearly identical mRNAs differing only in their 5' exons (Sample et al., 1989). The LMP-2A and -2B mRNAs encode for nearly identical proteins which share similar transmembrane domains and a 27 amino acid carboxy terminal domain (Kieff and Liebowitz, 1990). LMP-2B, however, lacks the entire 119 residue amino terminal cytoplasmic domain of LMP-2A.

Functionally, LMP-2 is a substrate for B lymphocyte src family tyrosine kinases, particularly lyn and fyn, and associates with a 70 kDa tyrosine phosphorylated cellular protein (Longnecker et al., 1991) (Burlhardt et al., 1992). While LMP-2 appears to play an important role during EBV infection in-vivo, its role in-vitro remains in question. Epstein-Barr virus recombinants carrying LMP-2A mutations, which do not express LMP-2A protein, are fully capable of initiating and maintaining B-lymphocyte growth transformation in-vitro (Longnecker et al., 1992). Surprisingly, expression of EBNA-1, EBNA-2 and LMP-1 are unaffected by the LMP-2A mutation. Importantly, BLCLs derived from the LMP-2A mutants are identical to wildtype EBV-transformed BLCLs with regard to growth characteristics, permisivity to lytic infection and virus replication. Thus, an important role for LMP-2 in-vivo is further supported by the expression of LMP-2 in latently infected B lymphocytes and NPC cells in-vivo, despite CTL activity against this protein. Recently, LMP-2 has been shown to
block the effects of surface Ig cross-linking on Ca^{++} mobilization and on lytic reactivation of EBV in latently infected B lymphocytes (Miller et al., 1995). LMP-2 appears to act as a constitutive dominant negative modulator of surface Ig receptor signaling through its effects on the Lyn and Syk protein tyrosine kinases, or regulators of these kinases. The effect of LMP-2 on preventing reactivation in-vivo likely contributes to immune escape and prevents destruction of the latent reservoir of EBV.

The most abundant EBV RNAs in latently infected B-cells are the EBV-encoded, small, nonpolyadenylated RNAs named EBER-1 and -2 (Howe and Shu, 1989). Unlike the other EBV genes expressed during latent infection, the EBERs are also transcribed during lytic infection. The majority of EBERs are localized within the cell nucleus where they are complexed with the cellular protein La (Howe and Steitz, 1986). Functionally, the EBERs have long been suspected of being involved in RNA splicing due to the long-range transcription and extensive splicing characteristic of latent infection, and because of the base complementarity of EBER-2 to known splice sites (Glickman et al., 1988). Realistically, this theory is incompatible with more recent evidence demonstrating that EBER expression during primary infection is delayed until after the splicing which is required for EBNA and LMP gene expression (Alfieri et al., 1991). Similarly, speculation of possible interferon effects of the EBERs are likely unfounded (Swaminathan et al., 1992).

The construction of EBV recombinants carrying EBER mutations has now helped to establish that both EBERs are dispensable for the in-vitro growth transformation of B-lymphocytes (Swaminathan et al., 1991). These EBER deletion mutants transform B-cells into BLCLs which are phenotypically identical to wildtype BLCLs in growth characteristics and ability to undergo lytic virus replication.

**EBV DNA Persistence in Latency.** During convalescence, low levels of virus are thought
to be maintained by sporadic replication in the epithelial and/or B-cells lining the oropharynx and latent virus persists in 1 in $10^{5-6}$ small resting B lymphocytes (Tosato and Blaese, 1985). Latently infected B cells typically contain between one and ten complete EBV episomes per cell (Adams and Lindahl, 1975) (Heller et al., 1981) and all EBV latently-infected cells express a minimum of the EBNA1 protein, which is required for episome maintenance and also likely required for episome amplification. However, while most EBV DNA persists in latently infected cells in an episomal form, the EBV genome is also known to integrate into chromosomal DNA (Matsuo et al., 1984) (Hurley et al., 1991). Evidence thus far indicates that integration is neither site specific nor a regular feature of EBV-mediated growth transformation. Since LMP-2 is the only EBV latent gene disrupted by linearization of the genome, the integrated form of EBV still retains it potential to transform B cells into permanently growing BLCLs. Clearly, the integrated form of EBV is limited in its ability to infect new cells, as episomal DNA is likely necessary for lytic cycle EBV replication, and lytic replication has not been reported in cells containing only the integrated form of EBV.

While BLCLs grown in-vitro express a restricted set of EBV latent genes (EBNAs-1, 2, 3A, 3B, 3C and LP, LMP-1 and 2, and EBER-1 and 2), EBV-infected resting B cells growing in-vivo express an even more restricted portion of the viral genome, limited to EBNA-1 (Miyashita et al., 1995) (Chen et al., 1995), and probably LMP-2a (Qu and Rowe, 1992) (Tierney et al., 1994). Following acute infection, EBV resides in small resting B cells which express a minimal number of B-cell activation markers or adhesion molecules and, largely for this reason, escape immune surveillance of the normal host. Thus, EBV transformed cells should be considered oncogenically transformed as they will proliferate indefinitely when cultured in vitro (Pope et al., 1969)(Henle et al., 1967)(Miller et al., 1969) and give rise to lymphoproliferative disorders including lymphoma in individuals with congenital (severe combined immunodeficiency; ataxia telangiectasia) or acquired (allograft recipients and AIDS) immunodeficiencies (Pultito et al., 1975)(Crawford et al., 1980) (Ziegler et al., 1982).
Lytic Infection/Virus Replication. The vast majority of latently infected B cells do not undergo lytic cycle replication, but can be induced to do so by activation with either phorbol esters, calcium ionophores or by crosslinking cell surface Ig (Luka et al., 1979) (Takada and Ono, 1989) (Sinclair et al., 1991). Following induction, cells undergo cytopathic changes characteristic of lytic herpesvirus infection, including chromatin margination, viral DNA synthesis, nucleocapsid assembly at the nucleus periphery, virus budding through nuclear membrane, and inhibition of host cell protein synthesis (Takagi et al., 1991).

In lytic EBV infection, immediate-early genes are defined as genes that are transcribed in newly infected cells in the absence of new viral protein synthesis. The key immediate-early trans-activators of EBV lytic cycle genes are the 1kb ZLF1 mRNA and the 2.8kb RLF1 RNA (Jenson and Miller, 1988) (Flemington and Speck, 1990). Transient expression of the ZLF1 ORF under the control of a heterologous promoter trans-activates two major EBV early gene promoters, HLF1 and DR, and the pattern of EBV early gene expression induced by ZLF1 is very similar to TPA-induced early lytic replication (Takada et al., 1986).

The induction of EBV lytic cycle replication results in increased episome copy number and suggests that circular episomal DNA replication is a precursor to subsequent DNA replication (Shaw, 1985). Unexpectedly, the EBV DNA polymerase is not required for viral DNA replication associated with episome establishment (Sixbey and Pagano, 1985).

The EBV genes expressed during the late stages of lytic infection are mostly structural viral proteins which permit virus development and egress. The viral glycoproteins are all late genes which have been extensively studied due to their potential importance in antibody-mediated immunity to EBV. The known glycoprotein genes are LLF1 gp350/220, BDLF3 gp150, ALF4 gp110, XLF2 gp85 and ILF2. ALF4 encodes a late 3.0 kb mRNA with a translated product of a 93 kDa protein, and when glycosylated is 110 kDa (Gong and Kieff, 1990).

Two EBV glycoproteins forming important parts of the virus coat are gp350/220 and
gp85 (Dolyniuk et al., 1976) (Beisel et al., 1985) (Heineman et al., 1988). Gp85 is a relatively minor virus component which is functionally involved in the fusion between virus and cell membranes (Miller and Hutt-Fletcher, 1982). Gp350/220 is the major virus coat glycoprotein and mediates virus binding to the B lymphocyte receptor CD21 (Fingeroth et al., 1984) (Nemerow et al., 1987) (Tanner et al., 1987). The finding that gp350/220 is the most abundant viral protein in lytically infected cell membrane has led to the hypothesis that high levels of gp350 may saturate CD21 so that newly released virus is not readorsbed to lytically infected cells.

Several potential strategies for inhibiting EBV replication are currently being developed, and most are based upon the unique interaction of EBV with B lymphocytes. Potential strategies include: 1) the dependence on gp350/220 interaction with CD21 for virus adsorption and penetration (Tanner et al., 1988), 2) preventing persistent infection by attempting to specifically block the interaction of EBNA1 with the ori-p site in EBV DNA, and 3) preventing the ZLF1 trans-activation of lytic virus replication by blocking recognition and interaction with cellular transcription factors.

Experimental Models of EBV Infection

A monumental breakthrough in our ability to study EBV infection came with the discovery that EBV can infect primary human B lymphocytes in vitro and transform them into permanently growing lymphoblastoid cell lines (Henle et al., 1967) (Pope et al., 1968). EBV-transformed B-cells will proliferate indefinitely in the absence of immune-mediated growth-regulation and not surprisingly, can be grown in nude mouse brain or in the peritoneum of SCID mice (Mosier et al., 1988) (Rowe et al., 1991) (Pisa et al., 1992). Latently infected B lymphoblastoid cells grown in vitro appear to phenotypically resemble the EBV tumors formed in SCID mice. In fact, EBV gene expression in SCID mice tumors is similar to BLCLs grown in vitro, albeit, EBNA2 expression is somewhat lower. Histologically, peripheral blood
lymphocyte (PBL)-derived hu-SCID tumors resemble the EBV+ large cell lymphomas that develop in immunosuppressed patients and, like the human tumors, are often located in multiple sites as individual monoclonal or oligoclonal foci (Rowe et al., 1991). These tumors develop with remarkable efficiency in the hu-SCID model and strongly suggest that lymphomagenesis involves the direct outgrowth of EBV-transformed B cells without the need for secondary genetic changes. It is therefore reasonable to assume that selection on the basis of cell growth rate alone is sufficient to explain the monoclonal/oligoclonal nature of these tumor foci. These findings are potentially very important as EBV+ large cell lymphomas in immunosuppressed individuals are likely to arise in a similar manner.

The contribution of individual EBV latent genes in maintaining growth transformation has been assayed by gene transfer into a variety of cells including primary or early passage continuous rodent fibroblasts and human B-lymphoma cell lines (Kieff and Liebowitz, 1990). EBNA-1, EBNA-LP and EBNA-3A have no apparent direct effect on primary or continuous rodent fibroblast growth alone or in cotransfection with other known oncogenes and have no effect on human B-lymphoma cell growth (Wang et al., 1987). EBNA-2 however, is directly involved in B-lymphocyte transformation, as two EBV strains (P3HR-1 and Daudi) deleted for EBNA-2 are unable to initiate growth transformation (Bornkamm et al., 1982) (Cohen et al., 1991). In spite of their inability to transform B cells (Miller et al., 1974), P3HR-1 and Daudi can still infect, replicate, and persist in a latent state in EBV-negative Burkitt's lymphoma cells. Construction of recombinant virus with the EBNA-2 mutant P3HR-1 and other defective EBV genomes, followed by selection of transformation competent EBV demonstrates the fundamental role played by EBNA-2 in that all transforming recombinants have a reconstituted EBNA-2 gene (Cohen et al., 1989). Additionally, gene transfer of EBNA-2 into Rat-1 cells has the effect of reducing serum requirements over that of Rat-1 control cells. Surprisingly, EBNA-2 has no transforming or immortalizing activity in primary rodent fibroblast cells alone or in combination with myc or ras (Kieff and Liebowitz, 1990). In gene
transfer experiments LMP-1 has been shown to impart potent transforming effects in continuous rodent fibroblast lines (Wang et al., 1985). In Rat-1 and NIH-3T3 cells, LMP-1 alters cell morphology and allows cell growth in medium containing markedly reduced amounts of serum. LMP-1 also causes loss of contact inhibition and loss of anchorage dependence in Rat-1 cells, enabling high efficiency cloning in soft agar. Additionally, Rat-1 cells expressing LMP-1 are tumorigenic in nude mice, whereas control Rat-1 cells are non-tumorigenic.

Initial attempts to develop a primate model for EBV infection were thwarted by the finding that old world primates are uniformly infected with and immune to a lymphocryptovirus closely related to EBV. However, new world primates are not uniformly infected with EBV related viruses and primary infection with EBV frequently results in death from acute lymphoproliferative disease (Shope et al., 1973). This form of infection provides an important model and studies of EBV-induced lymphomagenesis in cottontop tamarins have revealed that the tumors can be either monoclonal or oligoclonal large cell lymphomas closely resembling the EBV genome-positive B-cell lymphomas in human allograft recipients (Young et al., 1989). These tamarin tumors express at minimum, EBNA 1, EBNA 2, EBNA LP and LMP-1 as determined by immunohistochemistry and immunoblotting. Demonstration of EBNA 2 and LMP-1 expression in these tumors is particularly significant as it strengthens their resemblance to posttransplant lymphomas in humans. Furthermore, since EBNA-2 and LMP-1 are known to be required for EBV-induced B-cell growth transformation in vitro, their expression in these lymphomas constitutes some of the best evidence for a direct oncogenic role for EBV in vivo.

Clinical Manifestations & Immunobiology of EBV Infection

The propagation of EBV in man is dependent upon viral replication in the oropharynx and spread of virus to uninfected persons via contact with virus contaminated saliva (Gerber et al., 1972). In developing countries, primary infection with EBV typically occurs during the first several years of life, and is often sub-clinical. By contrast, in the western world where primary
infection with EBV is often delayed until the second decade, up to 50% of infections result in a self-limited constellation of signs and symptoms consistent with acute infectious mononucleosis, including fever, sore throat, lymphadenopathy, malaise, headache, atypical lymphocytosis, and splenomegaly (Henle and Henle, 1979). During the early stages of primary infection, large numbers of EBV infected B cells can be found in both peripheral blood and tissue, with as many as 10% of peripheral B cells being infected (Miller, 1990). Primary infection involves lytic replication in epithelial cells and latent infection of B cells which stimulate a profound cellular immune response, first by natural killer (NK) cells and closely followed by EBV-specific cytotoxic T cells and immunoglobulin secreting B lymphocytes (Tosato and Blaese, 1985) (Tomkinson et al., 1987) (Tomkinson et al., 1989) (Moss et al., 1992).

A hallmark of primary EBV infection is the appearance of “atypical lymphocytes” in the peripheral blood, which account for 60-70% of the total white cell counts averaging 12,000-18,000/mm³. The atypical lymphocytes are primarily activated T lymphocytes and NK cells, which secrete large amounts of inflammatory cytokines, believed to be the principle mediators of clinical symptoms during acute infectious mononucleosis (Niedobitek et al., 1992). A result of this intense immune response to the virus is a marked reduction in the number of latently infected B cells in the peripheral blood, which drop to around 1 in 10⁵-6 B cells during the subsequent months and remain at such levels indefinitely (Tosato and Blaese, 1985). This minute fraction of latently infected B cells serves as a reservoir for viral persistence or reactivation, and when grown in-vitro, these latently infected B cells can be propagated as B lymphoblastoid cell lines (BLCL). One pre-requisite to the efficient in-vitro expansion of latently infected B cells into BLCLs is the removal of EBV-specific CD8+ cytotoxic-T-cells or their inhibition by cyclosporin A (Bejarano et al., 1985). The virus-specific cellular immune response is long-lived in that EBV-specific CTL persist in the peripheral blood for life, likely a result of continued re-stimulation by EBV-antigen-bearing B-cells. These EBV-specific CTL bear unique receptors which recognize virus derived peptides in the context of self MHC class-I
molecules (Zinkernagel and Doherty, 1980). Prior to recognition by virus-specific CTL, endogenously synthesized viral proteins are first processed by cellular proteases to yield peptide fragments which bind self MHC class I molecules in the endoplasmic reticulum. Subsequently, the peptide-MHC complex is transported to the cell surface for presentation to virus-specific T cells. In the process of lysing EBV infected B cells, non-specific CTL binding to target cells is initiated by the interaction of cell surface adhesion molecules, including LFA-3 and ICAM-1 (Rodrigues et al., 1992) (Ybarrondo et al., 1994) (Teunissen et al., 1994) which are upregulated by LMP-1. Additional contributions to effector-target cell binding occurs between the CD8 molecule on T cells and MHC class-I molecules on target cells.

**Epidemiology.** Information on the geographic distribution of EBV and incidence of IM in different environmental and sociologic conditions has evolved through sero-epidemiologic studies of EBV antibody. EBV is a widely disseminated, relatively labile herpesvirus which is spread by intimate contact between susceptible persons and asymptomatic EBV shedders. For now known reasons, the majority of primary EBV infections throughout the world are subclinical and inapparent. Infectious mono is an easily recognized adult illness in societies with advanced hygiene standards, whereas the disease is a rarity in crowded, developing regions with lower standards of hygiene. For example, by 18 months of age, 82% of children in Ghana and Accra have experienced primary EBV infection as determined by EBV serology (Biggar et al., 1978). Many ascribe this finding to the intense personal contact and primitive personal hygiene as providing the opportunity for early acquisition and spread of EBV. Antibodies to EBV have been demonstrated in all population groups and worldwide, about 90-95% of adults are EBV-seropositive. The two strains of EBV (type-1 and 2), which differ in genomic sequence and ability to transform human B lymphocytes (Bornkamm et al., 1980) (Dambaugh et al., 1980) (Sample et al., 1990) are widely distributed geographically, while type-1 EBV is ten times more prevalent than type-2 in western countries, the ratio of type-1 to
type-2 EBV in African countries is only 2:1, and some individuals are co-infected with both types.

The incidence of clinically apparent infectious mononucleosis (IM) is greatest when primary exposure to EBV is delayed until the second decade of life. In the United States and Great Britain, EBV seroconversion occurs before age 5 in about 50% of the population, with a second wave occurring in the middle of the second decade. The overall incidence of IM in the U.S. is about 50 cases/10^5/yr, with the 15-24 year old age group having the highest incidence (Evans, 1969). No obvious yearly cycles or seasonal changes in incidence are known, as occurs with influenza.

Low titers of virus are present in throat washings of patients with IM and persist in the oropharynx for up to 18 months after clinical recovery. Interestingly, only 6% of IM patients recall previous contact with others having IM. Likewise, the ability to recover virus from throat washings is, not surprisingly, related to degree of immune compromise, with higher titers being present in more critically ill patients.

**Table: Frequency of EBV in throat washings in relation to degree of immune compromise**

<table>
<thead>
<tr>
<th>Patient Population</th>
<th>Frequency of EBV shedding</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV seronegative individuals</td>
<td>0%</td>
</tr>
<tr>
<td>Healthy EBV seropositive adults</td>
<td>12-25%</td>
</tr>
<tr>
<td>Solid tumor patients</td>
<td>27%</td>
</tr>
<tr>
<td>HIV-1 infected individuals</td>
<td>50%</td>
</tr>
<tr>
<td>Renal transplant patients</td>
<td>56-70%</td>
</tr>
<tr>
<td>Infectious mononucleosis patients</td>
<td>50-100%</td>
</tr>
<tr>
<td>Leukemia or lymphoma patients</td>
<td>74-92%</td>
</tr>
</tbody>
</table>

With regard to the impact of EBV on public health, in one study (Evans, 1960) infectious mononucleosis accounted for 5% of all student hospitalizations at the University of
Wisconsin, with an incidence of 450 cases/10^5 students/year. Across the country approximately 12-15% of susceptible college students undergo EBV seroconversion yearly (Evans et al., 1968), with hospital admission rates as high as 1449 cases/10^5 patients/year reported for Princeton University. Similar rates occur in the armed forces, and infectious mono ranks fourth as the cause of days lost to illness among army personnel.

**Diagnosis.** Distinction from other common viral infections depends on the complete triad of clinical, hematologic, and serologic determinations. In addition to the classic symptoms of fever, cervical lymphadenopathy, and pharyngitis, certain laboratory findings are necessary to confirm the diagnosis of IM. While heterophile antibodies and atypical lymphocytosis are present in 70-95% of new IM cases, EBV-specific antibodies can be demonstrated in virtually 100% of new cases (Lai, 1977). The IgM heterophile antibodies are present only during the initial stages of infection, in contrast to the IgG antibody against EBV structural proteins, which persist for the life of an individual (Henle and Henle, 1981).

<table>
<thead>
<tr>
<th>Clinical Diagnosis</th>
<th>Heterophile Antibody</th>
<th>Anti-VCA IgM</th>
<th>Anti-VCA IgG</th>
<th>Anti-EA-D IgG</th>
<th>Anti-EBNA IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>No EBV Exposure</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acute EBV Infection</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Previous Infection</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Serologic Diagnosis of Epstein-Barr Virus Infection**

**Histopathologic Changes.** Primary EBV infection in adults typically causes a self-limited, usually benign illness characterized by sore throat, fever and lymphadenopathy
lasting 2-3 weeks. During the acute phase, lymph nodes throughout the body are enlarged and contain increased numbers of large, moderately active lymphoid follicles. Germinal centers are also enlarged with increased numbers of blast cells, lymphocytes and histiocytes.

Splenomegally occurs in about one half of IM cases and autopsy studies have revealed that the organ is usually 2-3 times its normal weight. Histologically, the splenic capsule and trabeculae are edematous appearing and filled with lymphoid cells.

Not surprisingly, tonsillar biopsies have demonstrated intense lymphocyte proliferation with numerous mitoses. Additionally, bone marrow biopsies are usually not strikingly abnormal and hepatic histology is typically only mildly changed.

**Acute Infectious Mononucleosis**

**Clinical Manifestations.** Primary infection with EBV induces a self-limited illness lasting 2-3 weeks, which may present with a relatively broad spectrum of symptoms, several of which are almost always present with infectious mononucleosis (Schooley, 1995). The classical symptoms consists of pharyngitis, fever, and cervical lymphadenopathy (Hoagland, 1960). Onset may be abrupt, but more often is preceded by several days of prodromal symptoms, including anorexia, chills, sweats, headaches, myalgias, malaise and abdominal fullness. The most frequent complaint is sore throat, usually described as very severe.
Acute Infectious Mononucleosis

<table>
<thead>
<tr>
<th>Symptom</th>
<th>% Patients with Symptom</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sore throat</td>
<td>82</td>
<td>70-88</td>
</tr>
<tr>
<td>Malaise</td>
<td>57</td>
<td>43-76</td>
</tr>
<tr>
<td>Headache</td>
<td>51</td>
<td>37-55</td>
</tr>
<tr>
<td>Anorexia</td>
<td>21</td>
<td>10-27</td>
</tr>
<tr>
<td>Myalgias</td>
<td>20</td>
<td>12-22</td>
</tr>
<tr>
<td>Chills</td>
<td>16</td>
<td>9-18</td>
</tr>
<tr>
<td>Nausea</td>
<td>12</td>
<td>2-17</td>
</tr>
<tr>
<td>Abdominal Pain</td>
<td>9</td>
<td>2-14</td>
</tr>
<tr>
<td>Cough</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Emesis</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Arthralgias</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

The clinical manifestations of primary EBV infection are closely correlated with patient's age. While EBV infection in infants and children is often asymptomatic, adolescents and adults frequently experience symptoms of IM.

Some of the more common signs of acute IM are listed in the table below. Fevers to 38-39°C occur in over 90% of patients and typically resolve within 10-14 days. The tonsils are usually enlarged and the pharynx is erythematous with an exudate present in about 1/3 of cases. Symmetric, mildly-tender posterior cervical adenopathy is present in 80-90% of patients and splenomegaly can be appreciated in about 1/2 of patients if carefully sought.

Frequency of Signs Associated with Acute Infectious Mononucleosis

<table>
<thead>
<tr>
<th>Sign</th>
<th>% Patients with Sign</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphadenopathy</td>
<td>94</td>
<td>93-100</td>
</tr>
<tr>
<td>Pharyngitis</td>
<td>84</td>
<td>69-91</td>
</tr>
<tr>
<td>Fever</td>
<td>76</td>
<td>63-100</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>52</td>
<td>50-63</td>
</tr>
</tbody>
</table>

Humoral Immune Response to EBV Infection. Primary EBV infection induces circulating antibodies directed against viral antigens as well as unrelated antigens found on sheep and horse red cells. The latter antibodies, named heterophile antibodies, are a heterogeneous group of mostly IgM antibodies with either Forssman or Paul-Bruennell specificity, which do not cross-react with EBV antigens (Paul and Bunnell, 1932) (Paul and Bunnell, 1982). Serologically, the EBV-specific response represents a crucial component in the differentiation of primary EBV infection from other diseases presenting with lymphocytosis, fever, lymphadenopathy, and malaise. Diagnosis of EBV infection is based upon the transient serum levels of IgM against viral capsid antigens (VCA), as well as IgG against viral capsid antigens, early antigens (EA) and EBNA proteins (Henle et al., 1974) (Henle and Henle, 1981) (Okano et al., 1988). The transient IgM response to VCA reaches high titers early and disappears within weeks. IgM VCA antibodies are not demonstrable in the general population, and thus their presence is virtually diagnostic of acute EBV infection. IgG antibody titers to VCA reach peak levels two weeks later and thereafter persist at lower levels throughout life. Interestingly, IgG directed against early lytic cycle proteins (EA-D) tends to appear with the peak IgM response, and reaches maximal levels after the IgM response, significantly before the appearance of IgG to the structural proteins. Lastly, significant IgG anti-EBNA titers usually do not develop until well after primary infection. The following diagram depicts the onset and time course of peak anti-EBV Ig titers in relation to the corresponding clinical presentation during primary EBV infection.
Antibody Responses to EBV Antigens During Acute Infectious Mononucleosis.

![Graph showing antibody titers during the course of infectious mononucleosis.](image)

- **Densely hatched areas** represent ranges of antibody measured in normal hosts.
- **Finely hatched areas** represent ranges of antibody measured in immunocompromised hosts.

(Adapted from Henle, W: Human Pathology 5:551-565, 1974)

Gp350/220 is the most abundant viral protein in lytically infected cell plasma membranes and accordingly, most of the human EBV neutralizing IgG antibody response is directed against gp350/220 (Thorley-Lawson and Poodry, 1982) (North et al., 1982). These neutralizing antibodies reach maximal levels 6-7 weeks after the onset of illness and stable titers persist for life. Gp350-specific antibodies also mediate complement fixation and antibody-dependent cellular cytotoxicity (Khyatti et al., 1991). Fortuitously, the gp350 molecule is highly conserved between EBV strains 1 and 2 (Lees et al., 1993), and thus, gp350/220 is considered to be an essential component of any prospective EBV vaccine. In fact,
immunization with purified gp350/220 or infection with vaccinia expressing gp350/220 protects cottontop tamarins against a lethal, lymphomagenic EBV challenge (Morgan et al., 1988) (Morgan et al., 1988).

**Induction of EBV-Specific CTL.** The atypical lymphocytes which appear in the peripheral blood of acute IM patients between 1-3 weeks after onset of symptoms are primarily activated (HLA-DR+) CD8+ T-cells and also include CD16+ NK cells (Giuliano et al., 1974) (Johnsen et al., 1978) (Haynes et al., 1979) (Tomkinson et al., 1987). In 1975, several groups independently showed that when the high NK activity present in acute IM peripheral blood was removed from in-vitro T cell suspensions, there remained in-vivo activated T cells which specifically lyse EBV infected B cells independent of HLA restriction (Royston et al., 1975) (Svedmyr and Jondal, 1975). Importantly, the development of CD8+ T cell-mediated immunity against latently infected lymphocytes correlates closely with the decline of EBV-infected B cells from 1 in 10 during acute IM to 1 in 10^5-10^6 during convalescence (Thorley-Lawson et al., 1977) (Robinson et al., 1980) (Tosato and Blaese, 1985) (Miyashita et al., 1995). This latter finding is strengthened by in-vitro studies showing that EBV immune individuals posses virus-specific T cells which prevent the in-vitro outgrowth of EBV tranformed BLCLs (Rickinson et al., 1977) (Rickinson et al., 1979) (Rickinson et al., 1980). Initial efforts to demonstrate HLA class-I restriction of the EBV-specific T cells were hampered by the lack of a homogeneous population of effector cells used in 51Cr release assays (Lipinsky et al., 1979) (Seeley et al., 1981). Subsequently, EBV-specific T cell lines and clones were generated in-vitro by stimulation with autologous BLCLs in the presence of rIL-2, which lysed only EBV genome carrying cells in a HLA class-I-restricted manner (Sugamura and Hinuma, 1980) (Misko et al., 1980) (Rickinson et al., 1980) (Moss et al., 1981) (Rickinson et al., 1981) (Wallace et al., 1982). The success of expanding EBV-specific, HLA class-I restricted T cells in-vitro is heavily dependent upon repeated, selective stimulation of memory CTL at an appropriate responder to stimulator cell
ratio of 40:1 (Wallace et al., 1982) (Allen et al., 1982). Similarly, the in-vivo activated, non-HLA-restricted T cells originally described to be present during acute IM were subsequently shown to contain both EBV-specific, MHC class-I restricted CTL and a large non-HLA restricted population referred to as alloreactive CTL (Strang and Rickinson, 1987) (Strang and Rickison, 1987) (Tomkinson et al., 1989). Limiting dilution assays and regression plots have since estimated the precursor frequency of EBV-specific, memory cytotoxic-T-cells in the peripheral blood to be unusually high, at around 1/550 to 1/25,000 PBMC during convalescence (Rickinson et al., 1981) (Bourgault et al., 1991) (Carmichael et al., 1993).

Monoclonal antibody blocking studies have since confirmed the HLA-restricted nature of target cell lysis by EBV-specific CTL (Wallace et al., 1981). Specifically, antibodies directed against various MHC class-I alleles (W6/32, BB7.7) or β2-microglobulin (BBM1) on target cells block 75-85% of the CTL mediated lysis, while antibodies directed against common leukocyte antigens (HLe1, AC2) or HLA-DRw (TDR31.1) have no such effect. Additionally, antibody directed against CD8 (Leu 2a) on the EBV-specific effector CTL blocks the majority of target cell lysis (Wallace et al., 1982), while anti-CD4 antibody (Leu 3a) has no such effect. CTL mediated lysis of EBV-infected target cells can also be largely abrogated by antibody directed against the CD3 component of the T cell receptor (Tsoukas et al., 1982). Convincing evidence supporting the strict EBV antigen-specificity and HLA-restriction of EBV-specific CD8+ CTLs comes from work demonstrating that only EBV-containing, HLA-compatible cells can serve as "cold target competitors" to inhibit lysis of 51Cr-labeled autologous BLCLs (Tanaka et al., 1982). Interestingly, this same type of experimentation has led to the description of HLA-type-specific recognition (Gaston et al., 1983), whereby serologically indistinguishable HLA-A2 antigens encode differentially recognized T cell-restricting elements. Surprisingly, the serologically distinct HLA antigens HLA-A2 and HLA-Bw57 are capable of serving as cross-reacting restriction elements to a single CTL clone.

In contrast to the well characterized function of CD8+ CTL in eliminating EBV infected
cells, a similar role for CD4+ T cell-mediated lysis of EBV-infected B cells during acute infectious mononucleosis has not been established (Enssle and Fleischer, 1990).

The activated NK cells observed in the peripheral blood of acute IM patients are also seen with other viral infections and were first described in 1967 (Holm, 1967). NK cells are considered to play a primary role in the immune surveillance against virally or neoplastically modified cells, as they require no in-vitro stimulation or previous antigen exposure. Additionally, the cytotoxic activity of NK cells is known to be both spontaneous and IFN inducible (Landazuri et al., 1981). Interestingly, NK cells alone are not sufficient to counteract the establishment of EBV-transformed B cell lines in-vitro, however, they do contribute to improved BLCL regression in the presence of EBV-specific CTL (Masucci et al., 1983). This effect by NK cells may be due to either direct cytotoxicity or IFN production, as EBV and EBV-infected cells induce γ-IFN production by NK cells (Andersson et al., 1984), and α-IFN is known to inhibit EBV-induced B-cell proliferation in-vitro (Thorley-Lawson, 1981).

A concise summary of the major early advances in the study of host immune responses to EBV has been published (Sullivan, 1983), and provided the foundation for formulating the following integrated concept of human immunity to EBV.
More recently, efforts to identify the EBV-encoded CTL epitopes have been hampered by a lack of appropriate target cells in which to screen individual EBV latent genes. The same is not true for the study of other human viruses, as the majority of current research aimed at identifying T-cell epitopes encoded by viruses which infect humans continues to rely on EBV transformed BLCLs as a source of autologous target cells. The expression of EBV latent proteins in these cells preclude their use as targets in studies aiming to identify EBV CTL epitopes, the single exception being their use in studies characterizing EBV-type-specific T-cell epitopes (Moss et al., 1988) (Murray et al., 1990). A major advance in our ability to bypass this longstanding hurdle came with the development of EBV negative target cells consisting of
autologous human B cell blasts activated by ligation of cell surface IgM in the presence of IL-2 and IL-4 (Khanna et al., 1991). The construction of vaccinia virus recombinants encoding single EBV latent genes, used in conjunction with B cell blast targets, has allowed a relatively detailed analysis of EBV target antigens recognized by EBV-specific memory CTL (Khanna et al., 1992) (Murray et al., 1992) (Masucci et al., 1993). Results indicate that memory CTL from most EBV immune individuals recognize several of the eight known EBV latent proteins, with individual differences being largely accounted for by variation in each persons genetic makeup of HLA class-I alleles. Worthy of mention is the repeated absence of EBNA-1-specific memory CTL in published studies. A more detailed description of identified EBV CTL epitopes is included herein under the heading of "latent proteins recognized by CD8+ CTL".

**Apoptosis of EBV-Specific CTL in Acute IM.** The in-vitro expansion of previously in-vivo activated EBV-specific HLA-restricted CTL directly from the blood of infectious mono patients was first described in 1987 (Strang and Rickinson, 1987). These in-vivo activated CTL display several unusual features which differ significantly from the more familiar in-vitro activated memory CTL. Most significant is the need for immediate addition of IL-2 to the media used to expand in-vivo activated CTL. Unlike the procedure of stimulating memory CTL in-vitro, withholding IL-2 during initiation of an in-vivo stimulated culture results in apoptosis of the entire culture within 48 hours. This finding is in marked contrast to the in-vitro expansion of memory CTL, in which the early addition of IL-2 to the growth media results in the expansion of a population of cells which display non-specific anomalous T-cell cytotoxicity (Wallace et al., 1982). Several more recent studies have demonstrated that addition of exogenous IL-2 to growth media prevents the in-vitro apoptosis of primed CD45RO+ T cells from acute infectious mononucleosis patients (Uehara et al., 1992) (Akbar et al., 1993). These same activated, CD45RO+ T cells destined for apoptotic death can be identified using a mAb (IMN3.1) which recognizes a molecule intensely expressed on acute IM T cells, and only
dimly expressed on CD45RO+ memory T cells from normal individuals (Uehara et al., 1993). In keeping with the function of the proto-oncogene bcl-2 in blocking apoptosis (Korsmeyer, 1992), the absence of bcl-2 expression in activated CD45RO+ T cells from acute IM patients supports their increased susceptibility to programmed cell death (Tamaru et al., 1993). This latter finding also contrasts with the CD45RO+ memory T cells and naive CD45RA+ T cells from normal individuals which express much higher levels of bcl-2. The significance of this activation-driven depletion of mature T cells may partially explain our observed differences in primary vs memory T cell responses (described in the results section). One group has suggested that the low bcl-2 levels found in activated CD45RO+ T cells can be reconciled with studies demonstrating that T cell memory responses in-vivo are dependent on restimulation with the original priming antigen (Gray and Matzinger, 1991), which increases bcl-2 expression (Akbar et al., 1993). Thus, the familiar activation driven transition of naive CD45RA+ cells into activated CD45RO+ T cells (Clement, 1992), is accompanied by reduced bcl-2 expression, necessitating continued antigen stimulation for survival.

Lymphoproliferative Diseases

The principle biologic function of EBV that accounts for its role in the pathogenesis of lymphoproliferative diseases lies in the ability of the virus to induce unlimited proliferation of B lymphocytes, otherwise known as immortalization. Specific cell types susceptible to this EBV induced transformation include mainly well-differentiated, IgM+, resting B-lymphocytes which have undergone VDJ rearrangement and heavy-chain class switching (Brown et al., 1985). The efficiency of the immortalization process by EBV is quite high, and the percentage of cells susceptible to transformation has been estimated at 10% of all B cells (Sugden and Mark, 1977) (Tosato, 1991).

The immortalization process consists of two major phases, the first being initial B-cell
activation, which is triggered by attachment of the virus to the cell surface. The cell is activated to leave the G<sub>0</sub> phase and enter the G<sub>1</sub> phase, and is accompanied by expression of activation molecules such as Blast-2 (CD23) (Thorley-Lawson and Mann, 1985) (Thorley-Lawson et al., 1985). The second phase of immortalization consists of the establishment of a state of permanent blastogenesis requiring the expression of EBV gene products.

The importance of understanding the immune mechanisms responsible for the control of EBV induced B cell proliferation in man is underscored by an ever growing list of EBV-associated lymphoproliferative syndromes.

**X-linked lymphoproliferative syndrome.** The initial description of this syndrome came from studies involving three families in which an X-linked immunodeficiency to EBV resulted in fatal infectious mononucleosis in young male family members (Bar et al., 1974) (Provisor et al., 1975) (Purtilo et al., 1975). Most cases occur at around 2-5 years of age, and the common clinical course is that of an acutely fulminant process which initially resembles acute IM (Okano et al., 1991). The genetic mutation in the XLP syndrome has now been mapped, to the Xq25 region of the X chromosome (Skare et al., 1987). The genetic defect associated with XLP remains unknown but may be related to regulation of the intense CD8 T cell cytotoxicity initiated by acute EBV infection. Prior to EBV infection, males with the XLP mutation have normal cellular and humoral immune responses to bacterial and viral infections, with the exception of EBV. During primary EBV infection approximately 75% of affected males die with a fatal infectious mononucleosis syndrome, usually secondary to extensive liver destruction (Hamilton et al., 1980) (Sullivan and Woda, 1989). At autopsy, about 15% of individuals with fatal IM demonstrate a lymphoproliferative disorder described as immunoblastic sarcoma of B cells, Burkitt's lymphoma and other non-Hodgkins B cell lymphomas. In the 30% of affected males who survive primary EBV infection, all develop common variable immunodeficiency, characterized by recurrent infections, hypogammaglobulinemia, and 20-40% of these surviving
males develop lymphoproliferative disorders, including lymphoma (Sullivan et al., 1980) (Sullivan et al., 1983).

**Primary Immunodeficiency Diseases.** Shortly after the discovery of XLP, Epstein-Barr virus was recognized as a potential cause of severe infections and lymphoproliferative disorders in immunodeficient individuals. The medical literature is now replete with reports of fatal EBV-containing lymphomas developing in individuals with severe combined immunodeficiency following thymus transplants (Reece et al., 1980) (Hanto et al., 1981) (Crawford et al., 1980) (Crawford et al., 1981), and bone marrow transplants (Shearer et al., 1985).

**Burkitt’s Lymphoma.** Burkitt’s lymphoma is the most common childhood malignancy in equatorial Africa, and was first described in 1958 by the British surgeon Denis Burkitt (Burkitt, 1958). This unmistakable tumor typically presents in the jaws of young patients, and the majority of endemic cases occur in discrete geographic climates located along a belt across Africa lying 10° north and south of the equator (Burkitt, 1962). Since the initial description of the climatic distribution of this tumor, a biological vector was implicated in its etiology. Fortuitously, a pathologist, M.A. Epstein attended a lecture given by Dr. Burkitt in 1961, in which he described this bizarre cancer. With tumor biopsies provided by Dr. Burkitt, Epstein set about the task of searching for a virus to explain the etiology of this lymphoma. Three years later, Epstein and colleagues described a new herpesvirus present in most Burkitt lymphomas, now known as Epstein-Barr virus (Epstein et al., 1964).

Burkitt lymphoma is characterized by specific, reciprocal chromosomal translocations involving the c-myc locus on the long arm of chromosome 8 and one of the immunoglobulin loci on chromosome 14, 2 or 22 (Bornkamm et al., 1988). The juxtaposition of c-myc next to a heavy- or light-chain immunoglobulin promoter is a necessary although not sufficient step in the
development of BL. Additional factors are likely provided by EBV, as there is almost a universal association of EBV with the endemic form of Burkitt's lymphoma, thus lending much support to the tumorigenic potential of this virus (deThe et al., 1978). The ability of EBV to growth-transform human B cells (Pope et al., 1968), along with the potential to induce malignant lymphomas in new world monkeys (Shope et al., 1973) make EBV a strong candidate for contributing the remaining transforming factors in the multistep process of BL development. In fact, EBV infection precedes development of endemic BL as clonal EBV genomes exists in all tumor cells, implicating EBV's role in malignant conversion. While evidence for an etiologic role of EBV in BL is convincing, it is not yet conclusive (Epstein, 1978) (Henle et al., 1978).

Greater than 95% of endemic BL tumor cells contain copies of the EBV genome (Ziegler, 1981) (Miller, 1990), while only 15-20% of sporadic (outside high incidence areas) cases of BL contain EBV genomes (Burkitt, 1967). Recent findings suggest this latter figure may be as high as 45% (Razzouk et al., 1996). Analysis of the EBV genome terminal repeat frequency in endemic Burkitt lymphomas has shown that the tumors originate in the lineage of a single EBV-infected B cell (Raab-Traub and Flynn, 1986) (Brown et al., 1988) (Neri et al., 1991).

Initial attempts to define the cell surface phenotype of BL tumors were hampered by the inconsistent characteristics of in-vitro grown BL cells (Favrot et al., 1986). Subsequent work demonstrated that fresh tumor biopsies consistently displayed a homogeneous surface phenotype including the pan B cell marker CD20, the common acute lymphoblastic leukemia antigen (CALLA, CD10), and the BL-associated antigen (CD77) (Rooney et al., 1986). Additionally, unlike in-vitro grown BLCLs, fresh BL biopsy cells do not express the B cell activation antigens CD23, CD30, CD39, and CD70 and the cell adhesion molecules LFA-1 (CD11a/18), ICAM-1 (CD54), and LFA-3 (CD58) (Gregory et al., 1988). The fresh BL tumor biopsies which retain this resting B cell phenotype (latency type-I) also differ from BLCLs (latency type-III) in the expression of EBV latent genes (Rowe et al., 1986) (Rowe et al., 1987). While in-vitro propagated BLCLs typically express six EBNA proteins and two latent
membrane proteins, newly explanted BL cells typically express only EBNA-1 (Gregory et al., 1990) (Sample et al., 1991). This tightly restricted EBV latent gene expression in BL cells, is likely the result of inactivity of the Wp and Cp promoters in BL cells (Jansson et al., 1992), and the use of a newly described EBNA-1 specific promoter (Fp/Qp) located in the BamHI-F/Q fragment of the genome (Sample et al., 1991) (Schaefer et al., 1991). Interestingly, the serial passage of endemic BL cell lines (type-I) in-vitro results in the transition to a more BLCL-like phenotype (type-III), associated with the full spectrum of EBV latent gene expression resembling BLCLs (Rowe et al., 1987) (Gregory et al., 1990). BLCLs and BL cells grown in-vitro can also be induced to switch from one form of latency to another depending upon culture conditions. The limited expression of EBV latent genes in BL cells, along with reduced expression of MHC class-I antigens (Masucci et al., 1987) and lack of adhesion molecules likely contributes significantly to the ability of these cells to escape T cell-mediated destruction (Rooney et al., 1985).

Recently, a fourth form of EBV latency been described in tumor cells of smooth muscle origin (leimyosarcomas) occurring in young people with AIDS and organ transplant recipients (McClain et al., 1995) (Lee et al., 1995). This new form of EBV latency (type-IV) is characterized by EBV latent gene expression restricted to EBNA1 and 2, suggesting that immunosuppression plays a major role in the pathogenesis of these tumors which express the immunogenic EBNA2 protein. The following diagram demonstrates the more restricted forms of EBV latency (type-I & II) observed \textit{in vivo} (top), as compared to the less restricted forms of latency (type-III) displayed by most EBV infected cells propagated \textit{in vitro} (bottom), in the absence of immune regulation.
Types of EBV Latency in Normal and Neoplastic Cells

**Latency in-vivo**

- Burkitt's Lymphoma (type-I)
- Latently Infected B cell (type-I)
- Hodgkin's Lymphoma (type-II)

**Latency in-vitro**

- Latent Type III
- Latent Type III
- Latent Type II

**Latent Gene Expression**

- EBNA 1
- EBNA 1 +/- LMP2
- EBNA 1

Post-transplant Lymphoproliferative Disease. Virus-specific CTL are unable to eliminate EBV from the body, yet they are essential in preventing EBV-mediated B-lymphoproliferation. A variety of immunocompromised states are associated with increased numbers of EBV-infected B cells in the peripheral blood, which can progress to polyclonal masses or even monoclonal lymphomas. The incidence of posttransplant EBV lymphomas rises sharply with the degree of immunosuppression, and are encountered all too often in persons with secondary immunodeficiency such as that due to chronic immunosuppression to prevent
allograft rejection (Hanto et al., 1985). Phenotypically, most post-transplant lymphomas are polyclonal, immunoblastic and often resemble the type-III latency described for BLCLs, and accordingly, express all or most EBV latent genes, B cell activation markers, and cell surface adhesion molecules. EBV-induced lymphoproliferation has been described in immunocompromised renal (Crawford et al., 1980), cardiac (Bieber et al., 1984), and liver allograft recipients (Starzl et al., 1980), and recipients of bone marrow (Sullivan et al., 1978) and thymic epithelium transplants (Reece et al., 1980). In recipients of bone marrow transplants, EBV-associated lymphomas are generally confined to recipients of T-cell-depleted allografts. Fortunately, these EBV+ donor-derived immunoblastic lymphomas have recently been shown to regress when recipients are treated with leukocytes derived from the original marrow donors (Papadopoulos et al., 1994) (Rooney et al., 1995) (Heslop et al., 1996).

Approximately 6% of individuals receiving renal allografts develop de novo malignancy (Penn, 1978) (Hanto et al., 1981). It is now recognized that renal allograft recipients receiving cyclosporin-A immunosuppressive therapy are at increased risk for developing malignant lymphoma, likely a result of cyclosporin-mediated inhibition of long-term T cell memory to EBV (Crawford et al., 1980). Support for this hypothesis comes from studies demonstrating the ability of cyclosporin-A to promote the outgrowth of EBV-transformed B cell lines in-vitro (Bird et al., 1981) (Rickinson et al., 1984). Still more convincing is the observation that post-transplant lymphomas frequently regress upon discontinuation or reduction of immunosuppressive therapy, often without subsequent graft rejection (Starzl et al., 1980). The development of lymphomas in immunosuppressed individuals also extends to the use of immunosuppressive therapy in the treatment of rheumatic disorders. Several studies have reported an increased risk of large-cell lymphoma in patients with rheumatic disease who were treated with azathioprine or cyclophosphamide (Kinlen, 1985). Similarly, two patients developed EBV-associated malignant lymphomas during methotrexate treatment, and in each case the tumor regressed following the withdrawal of
methotrexate (Kamel et al., 1993).

AIDS & Non-Hodgkin's Lymphoma. In 1982, prior to the identification of HIV, four cases of Burkitt's-like lymphoma (undifferentiated, monoclonal B cell) were described in homosexual men in San Francisco (Ziegler et al., 1982). The immunoblastic monoclonal, Burkitt-like phenotype of these tumors allowed for easy distinction from the EBV-induced polyclonal, immunoblastic lymphomas which occur in allograft recipients. Since 1982, EBV-induced lymphoproliferative disorders and non-Hodgkin's lymphomas (NHL) have been shown to occur approximately 60-100 fold more frequently in the setting of immunodeficiency associated with HIV infection (Beral et al., 1991) (Levine, 1992). In fact, a larger study conducted in Los Angeles county from 1984-1992 showed that EBV was associated with 39 of 59 (66%) HIV-related systemic lymphomas (Shibata et al., 1993). Analysis of EBV terminal repeats in these lymphomas again confirmed their monoclonal origin, and c-myc rearrangements were noted in 40%. Evaluation of EBV latent gene expression in the HIV-related lymphomas revealed a unique pattern of expression, restricted to EBNA-1 and LMP-1, not previously described in B cells.

Given the profound immune defects in HIV-infected patients, along with the known role of CTL in controlling EBV-induced proliferation, it is not surprising that the number of EBV-infected B cells in the peripheral blood of those with AIDS is higher than the general population (Birx et al., 1986).

Infection with HIV is also associated with a markedly increased risk for developing non-Hodgkin's lymphoma, which increases still further with longer survival and declining CD4 counts (Beral et al., 1991). AIDS associated NHL, usually of B cell origin, is a relatively late manifestation of HIV infection (Levine, 1990), and is now considered an AIDS defining condition (Ziegler et al., 1988). However, unlike the monoclonal Burkitt's type lymphomas, AIDS related NHL ranges from polyclonal lymphoproliferation to monoclonal lymphoma, and
is not uniformly EBV associated (McGrath et al., 1991). For unknown reasons, the majority of EBV-associated NHL in AIDS patients have presented as primary CNS lymphomas (Shiramizu et al., 1992).

**Hodgkin’s Disease.** EBV genomic DNA was first reported in Hodgkin’s disease in 1987 (Weiss et al., 1987). Recent evidence supports a strong role for EBV in the pathogenesis of HD, where EBV genomes have been detected in tumor material in 19-50% of HD cases, and in situ hybridization has localized the EBV DNA to the malignant Hodgkin’ Reed-Sternberg cells. EBV appears to be more tightly linked to "Western" HD cases, where the genome is present in up to 50% of cases (Pallesen et al., 1991). The reported presence of monoclonal EBV genomes in HD suggests that EBV infection preceeds clonal cellular proliferation. Expression of the small EBV-encoded RNAs, EBER1 and EBER2, is a consistent finding of EBV associated HD, and EBNA1 protein can be detected in >70% of these cases (Grasser et al., 1994). As with Burkitt’s lymphoma, the association of EBV with HD appears to vary geographically, as 94% of classical HD occuring in Peru contain EBV transcripts within the Reed-Sternberg cells (Chang et al., 1993). Healthy western populations are infected with predominantly type-1 EBV (Sixbey et al., 1989), and not surprisingly, the majority of EBV detected in HD is type-1 (Gledhill et al., 1991). In contrast, there is an almost equal frequency of type-1 and type-2 EBV in HIV-associated NHL (Boyle et al., 1991) and endemic Burkitt’s lymphoma (Young et al., 1987). The type of EBV latency in HD has been evaluated by PCR and in-situ hybridization of EBV specific latent gene transcripts, and most resemble the type-II latency described in nasopharyngeal carcinoma (Deacon et al., 1993) (Grasser et al., 1994). In Hodgkin’s lymphomas, EBNA-1 is expressed from the BamHI Q promoter, as in NPC and BL, while expression of other EBNAs is prevented by the silent BamHI Cp and Wp promoters. LMP-1 and LMP-2 transcripts are consistently detected in these EBV-positive Hodgkin’s lymphomas, irrespective of the histologic subtype. However, current information suggests the link between
EBV and HD may apply to only certain histologic subtypes, as the frequent presence of EBV in HD is usually associated with only the mixed cellularity and nodular sclerosing subtypes.

While the majority of EBV associated Hodgkin's disease tumors express several immunogenic viral proteins, cellular immunity against EBV is often impaired in this setting. Curiously, both LMP1 and LMP2 contain known CTL epitopes (Khanna et al., 1992) (Murray et al., 1992) and would be expected to facilitate immunologic control of HD tumors expressing these proteins. In one study, HLA class I-restricted EBV-specific CTLs were demonstrated in 3 of 3 EBV+ tumors, whereas cultures from 6 of 6 EBV+ tumors were either noncytotoxic or displayed only LAK-type activity (Frisan et al., 1995). In evaluating the EBV-specific reactivities present in tumor biopsies, a high prevalence of CD4+ cells (CD4/CD8 >3) was detected in half of the EBV+ cases but only 1 of 5 EBV- HD cases. While general impairment of EBV-specific CTL responses is not a general feature of HD, these findings suggest that tumor associated suppression of EBV-specific CTL responses may play a major role in the pathogenesis of EBV+ HD tumors. One possible explanation for the impaired CTL activity in this setting may be the result of HD tumor cells expressing viral antigens which deliver inhibitory signals, and render EBV-specific T cells anergic. However, unlike the phenotype of latency type 1 cells, these Hodgkin's RS cells have many characteristics of professional antigen-presenting cells, including high levels of the costimulatory molecule B7 (Harding et al., 1992).

**EBV Associated T Cell Lymphomas.** EBV-associated T cell lymphoproliferative disorders are now being recognized more frequently, including EBV-associated T-cell lymphomas (Su et al., 1991) (Chen et al., 1993), and EBV-infection of both neoplastic and non-neoplastic T-lymphocytes in virus-associated haemophagocytic syndrome (Kawaguchi et al., 1993). Analysis of EBV expression indicates that EBV-infected T-cell lymphomas represent clonal expansions of single EBV-infected T cells with a pattern of gene expression distinct from Burkitt's lymphomas or posttransplant lymphomas but similar to EBV expression in
nasopharyngeal carcinomas. Similarly, a monoclonal expansion of EBV-infected T-cells occurs in EBV-associated haemophagocytic syndrome, and the EBV-infected T cells represent a range from preneoplastic to overtly malignant proliferations.

Non-Lymphoid EBV Diseases

Nasopharyngeal Carcinoma. Worldwide, nasopharyngeal carcinoma is relatively rare, however it is one of the most common cancers in southern China with age-adjusted incidence rates of up to 55 per 100,000 (Ho, 1978). However, unlike Burkitt's lymphoma, the association of EBV with NPC is highly consistent in both low- and high-incidence areas (Andersson et al., 1977), in fact, EBV is present in every anaplastic nasopharyngeal carcinoma cell.

A wealth of new evidence continues to strengthen the role of EBV as the primary etiologic agent in the pathogenesis of nasopharyngeal carcinoma (Pathmanathan et al., 1995). The presence of a single clonal form of EBV in preinvasive lesions such as nasopharyngeal dysplasia or carcinoma in situ, indicates that EBV-induced cellular proliferation precedes the acquisition of invasiveness of these tumors. Nasopharyngeal carcinoma cells express a specific subgroup of EBV latent proteins, including EBNA-1 and two integral membrane proteins, LMP-1 and LMP-2, along with the BamHI-A fragment of the EBV genome (Brooks et al., 1992) (Busson et al., 1992). While EBNA-2 is essential for the transformation of lymphocytes, its absence in preinvasive neoplasia and nasopharyngeal carcinoma indicates that EBNA-2 is not required for altered epithelial cell growth (Young et al., 1988). In light of the newly described molecular link between LMP-1 and cell growth (Mosialos et al., 1995), the universal presence of LMP-1 in NPC makes it a likely prerequisite to this multistep neoplastic transformation. Briefly, LMP-1 is known to mimick activated receptors by aggregating in the plasma membrane, and allowing the cytoplasmic tail to interact with and activate TRAF3, a TNF-associated factor involved in growth signaling. A compilation of the evidence supporting this theory is
summarized in a recent editorial (Kieff, 1995).

The significance of identifying a link between precursor lesions and EBV infection is underscored by realizing that current efforts to improve immunity to EBV-infected cells may eventually be used to prevent the development of nasopharyngeal carcinoma.

**Smooth Muscle Tumors, Aids & Liver Transplantation.** In addition to malignant lymphomas, children with AIDS experience an unusually high incidence of smooth muscle tumors (leiomyomas and leiomyosarcomas) (Chadwick et al., 1992). Ordinarily, the incidence of leiomyomas in children is extremely low, however, the recent demonstration that EBV can infect smooth-muscle cells in AIDS patients, may help explain how EBV contributes to the pathogenesis of these leiomyomas (McClain et al., 1995). Still further convincing evidence for an etiologic role of EBV in the development of neoplastic lesions is provided by the recent description of smooth-muscle tumors containing clonal EBV, developing in three children after liver transplantation (Lee et al., 1995).

**Oral Hairy Leukoplakia.** Another EBV-induced disease in adult AIDS patients is oral hairy leukoplakia (OHL), an unusual wartlike disease of the lingual squamous epithelium. Virus replication is evident only in the upper layers of the epithelium, and is effectively inhibited by acycloguanosine. Interestingly, the OHL lesions appear to be relatively specific for HIV-related immunodeficiency, as OHL is only rarely observed in patients with other immunodeficiencies (Epstein et al., 1991).
Patterns of EBV Gene Expression In Normal and Neoplastic Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>In-vivo/In-vitro</th>
<th>Latency Type</th>
<th>EBV Gene Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burkitt's Lymphoma</td>
<td>In vivo</td>
<td>I</td>
<td>EBNA-1</td>
</tr>
<tr>
<td>Burkitt's Lymphoma</td>
<td>In vitro</td>
<td>III</td>
<td>EBNA1, 2, 3A, 3B, 3C, LP LMP-1, 2</td>
</tr>
<tr>
<td>Lymphoblastoid Cell Line</td>
<td>In vitro</td>
<td>III</td>
<td>EBNA1, 2, 3A, 3B, 3C, LP LMP-1, 2</td>
</tr>
<tr>
<td>Peripheral Blood B-Cell</td>
<td>In vivo</td>
<td>I</td>
<td>EBNA-1, +/- LMP2</td>
</tr>
<tr>
<td>Nasopharyngeal Carcinoma</td>
<td>In vivo</td>
<td>II</td>
<td>EBNA-1, LMP-1, 2</td>
</tr>
<tr>
<td>Hodgkin's Lymphoma AIDS-NHL</td>
<td>In vivo</td>
<td>II</td>
<td>EBNA-1, LMP-1, 2</td>
</tr>
<tr>
<td>Post-Transplant Lymphoma</td>
<td>In vivo</td>
<td>III</td>
<td>EBNA1, 2, 3A, 3B, 3C, LP LMP-1, 2</td>
</tr>
<tr>
<td>T-Cell Lymphoma</td>
<td>In vivo</td>
<td>II</td>
<td>EBNA-1, LMP-1, 2</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>In vivo</td>
<td>IV</td>
<td>EBNA-1, 2</td>
</tr>
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</table>

EBV-specific cytotoxic-T-cells

Phenotype of effector cells. The most likely route of primary EBV infection is via the oropharyngeal epithelium, where virus can also infect susceptible B lymphocytes within the pharyngeal lymphoid tissue (Sixbey et al., 1984). During the initial 30-50 day incubation period, EBV replication and dissemination occur throughout the lymphoreticular system and are accompanied by attendant immune aberrations. Early in the course of illness, non-EBV-specific cell-mediated immunity is depressed, as measured by cutaneous anergy and depressed proliferative responses to antigens and mitogens (Mangi et al., 1974).

During the first few weeks of clinical illness, an impressive mononuclear lymphocytosis is present which consists primarily of activated, cytotoxic-T-lymphocytes (Rickinson, 1986)
Long after the clinical symptoms of infectious mono subside, CD8+ virus-specific memory-T-cells remain in the peripheral blood of EBV immune individuals and retain the ability to suppress the outgrowth of autologous EBV-transformed B-lymphocytes (Svedmyr and Jondal, 1975) (Thorley-Lawson et al., 1977) (Moss et al., 1978). Corresponding T cell preparations from EBV seronegative donors have no such activity. Confirmation of the T cell identity of the EBV-immune lymphocytes comes from studies demonstrating that PBMC from acute IM donors, when depleted of NK activity by erythrocyte-Ab rosetting, preferentially lyse chromium-labeled autologous lymphoid cell lines carrying the EBV genome (Royston et al., 1975). Cytotoxic T cell recognition of EBV-infected cells is known to be MHC class-I restricted (Moss et al., 1981), having been confirmed in blocking studies involving mAb to HLA determinants (Wallace et al., 1981). Surprisingly, while the virus-specific T-lymphocytes remain for the life of an individual (Rickinson et al., 1981), this profound and stable immune response is still insufficient to totally eliminate virus from the body. In fact, EBV-induced lymphoproliferative disease is only rarely witnessed in the human host, attesting to the powerful immune control over this potentially oncogenic virus, which at the same time permits life-long EBV persistence. The impressive biologic properties of EBV underscore our need for a more thorough investigation of the target structures recognized by the cytotoxic T cells which mediate surveillance against EBV induced lymphoproliferative disorders. In this sense, infectious mono represents the ideal model system to study the balance between EBV and the human host, which usually culminates in successful control of the virus. Unfortunately, there is no EBV vaccine available for use in humans.

**Latent proteins recognized by CD8+ memory CTL.** The discovery of antigen-specific T cells directed against a virus with the ability to induce malignant B cell proliferations in man prompted early efforts to identify the postulated lymphocyte detected membrane antigen (LYDMA) target structure expressed on EBV-transformed B lymphocytes (Moss et al., 1981). A
major advance in our ability to study EBV immunity came from the discovery that EBV-specific T-lymphocytes can be expanded into cell lines or cloned in-vitro by direct stimulation with autologous EBV-infected B lymphocytes and recombinant IL-2 (Svedmyr et al., 1974) (Sugamura and Hinuma, 1980) (Rickinson et al., 1981) (Wallace et al., 1982) (Slovin et al., 1983) (Bishop et al., 1985) (Moss et al., 1992). The majority of EBV-specific cytotoxic-T-lymphocytes are CD8+, and thus express T-cell-receptors (TCR) which recognize viral peptides in the context of self MHC class-I molecules. Accordingly, in-vitro restimulated effector T cells have been extensively utilized as immunologic probes to identify the EBV-encoded molecular components of LYDMA.

Early efforts to identify LYDMA focused on the latent membrane protein LMP-1, including one study in which a 10 amino acid peptide (residues 43-53) from LMP-1 was demonstrated to specifically stimulate EBV-specific CTL (Thorley-Lawson and Israelson, 1987). In a subsequent study, a less well defined region of LMP-1 was determined to contain at least one CTL epitope (Murray et al., 1988). In the latter case, target cells were derived by expression of the individual EBV latent genes EBNA-1, 2, LP and LMP-1 in the EBV negative BL, Loukes. These investigators clearly demonstrated the MHC restriction of the CTL as only LMP expressing cells carrying the HLA-A2 or B44 class I alleles were recognized as targets in chromium release assays. Additionally, a truncated version of LMP-1 (Tr-LMP), missing the amino terminal 128 amino acids, was not recognized in this same system, suggesting an amino terminal location for this LMP-1 epitope.

At around the same time, EBV-specific CTL were shown to be capable of precise immunologic discrimination between target cells expressing EBNA proteins derived from either type-1 or 2 EBV strains (Moss et al., 1988) (Misko et al., 1991). Based upon differential recognition of EBV-transformed B lymphocytes, the EBV latent proteins EBNA-2, 3A, 3B and 3C were shown to contain EBV type specific epitopes, which likely explain the reported EBV type-specific T-cell responses (Rowe et al., 1989). In order to more effectively identify the
latent proteins responsible for this T-cell-mediated type-specific-recognition, effector T cell clones established by stimulation with autologous BLCLs were tested for their ability to lyse B cell blasts infected with recombinant vaccinia constructs expressing single EBV latent genes or incubated with EBNA peptides. The EBV-specific CTL clones recognized type-specific epitopes within EBNA-2, 3A and 3C, as well as type-common epitopes within EBNA-2, 3A, 3C, LP and LMP-1 (Khanna et al., 1991) (Khanna et al., 1992). The EBNA-3A and 3C proteins were recognized most frequently (11/14 donors) by the CTL clones while no CTL epitopes were localized within EBNA-1. Interestingly, the specificity of a large number of EBV-specific T cell clones (68%) could not be identified, suggesting the need to screen a more comprehensive panel of EBV latent proteins for CTL epitopes. The immunodominance of the EBNA3 family of latent proteins described for these EBV-specific CTL clones has also been confirmed with polyclonal EBV-specific T cell lines (Murray et al., 1992). In this latter study, EBV-specific CTL from 14/16 donors recognized target cells expressing EBNA-3C. It is worth pointing out that both EBNA-3B and LMP-2 sensitized target cells to EBV-specific CTL-mediated lysis, suggesting that all latent proteins except EBNA-1 serve as targets for CTL recognition.

This same group had earlier examined the cytotoxic T cell responses in two other EBV immune donors (Murray et al., 1990). The EBV latent proteins EBNA-2, 3A, 3B and LP were expressed in target cells under the control of the vaccinia virus p7.5 promoter, and two donor CTL responses were mapped to epitopes in EBNA-2 and EBNA-3A. In retrospect, this work established an important experimental approach that is now being extended to all of the EBV latent proteins in an effort to identify the immunodominant EBV epitopes recognized within the general population.

One the first EBNA-encoded CTL epitopes identified was described by Burrows, who tested 76 peptides derived from type-1 EBV latent proteins EBNA-2, 3A, 3B and 3C for their ability to sensitize type-2 BLCLs to lysis by an EBV type-1-specific CTL clone (Burrows et al., 1990). One 25 amino acid peptide of the 76 tested, (residues 329-353) from EBNA-3A, was
recognized in the context of HLA-B8. In a similar series of experiments 58 peptides derived from the EBV latent proteins EBNA-2, 3A, 3B and 3C were tested for their ability to sensitize target cells to lysis by a panel of EBV-specific CTL clones. In this case, one type-common peptide derived from EBNA-3C was identified, which was recognized in the context of HLA-BW44 (Burrows et al., 1990).

EBV derived oligopeptides have also been used to induce secondary cytotoxic T cell responses in-vitro (Schmidt et al., 1991). Three peptides derived from EBV latent proteins EBNA-2 (QLS DTPLIPT IFVGENTGV), EBNA-3A (TETA QA WNFGRGKGYGIDLLRTE) and EBNA-3C (EEN LLDFVRFMGVMSSCNNP) sensitized PHA stimulated blasts to lysis by EBV-specific CTL. The EBNA-2 epitope was recognized in the context of HLA-B8, while the restriction elements for the other two peptides were not determined.

In a series of peptide screening experiments conducted by Misko et al., an immunodominant EBV-specific CTL epitope, represented by peptide 68 from the type-1 virus, was mapped to EBNA-3A and recognized in the context of HLA-B8. The authors further demonstrated that peptide 68 was not expressed as a functional CTL epitope by type-1 LCLs infected with the EBV B95-8 isolate. This finding points to a major weakness inherent in trying to identify CTL epitopes by screening panels of virus encoded peptides synthesized in vitro. Although capable of being recognized by CTL clones, these peptides do not necessarily represent the naturally processed, biologically relevant peptides presented in vivo (Misko et al., 1991). Such peptide based vaccine strategies, while capable of inducing an immune response, would not necessarily be immunologically relevant and would serve no actual protective function against the pathogen.

Perhaps the most significant changes in our understanding of cell-mediated immunity to EBV comes from the work described in this thesis and that recently presented by other groups. Our previously described results (Beaulieu and Sullivan, 1994), now expanded to include the evaluation of 35 acute IM patients demonstrate that while the virus-specific primary CTL
response is broadly directed against the full spectrum of latent proteins, including EBNA1, the memory CTL response, which essentially lacks EBNA1 reactivity, is directed primarily against the EBNA 3 proteins (3A, 3B, 3C). CTL from 7 of the 35 acute IM patients evaluated recognized EBNA1 expressing targets, and in 4 of these 7 patients, EBNA1 was an immunodominant antigen. The detection of only one EBNA1-specific memory CTL response among the 32 patients tested may help explain how latently infected B cells in-vivo, expressing only EBNA1, escape CTL recognition and thus might serve as a reservoir for viral persistence and/or reactivation. Interestingly, since our initial description of EBNA-1 specific CTL, another group has subsequently described a CTL clone which recognizes a MHC class-II restricted CTL epitope within EBNA-1 (Khanna et al., 1994), however, the EBNA1-clone does not lyse the autologous BLCL. Thus, the in-vivo role of this clone is unknown, but initial indications suggest the presence of significant numbers of these memory T-cells specific for this EBNA-1 epitope.

To date, the search for EBV-encoded CTL epitopes has focused primarily on EBV antigens expressed in BLCLs, using memory CTL reactivated in-vitro. The development of an effective subunit vaccine will require knowledge of CTL responses in acute as well as latent viral infections, and necessitate that we determine whether EBV structural proteins contain important CTL epitopes relevant to immune control during primary infection. The significance of recent findings are discussed in the context of EBV persistence and immune escape.

**EBV Vaccines**

The multitude of evidence implicating EBV in the etiology of a variety of human neoplasms has made appealing the prospect of developing a viral-based vaccine effective against human cancers. In endemic regions of the world, vaccination of infants against EBV would potentially reduce the incidence of Burkitt's lymphoma, as well as nonendemic Hodgkin's lymphoma, NPC, EBV-associated T cell lymphomas and leimyosarcomas, while
vaccine administration in developed countries would prevent the development of acute IM in young adults. With an annual U.S. incidence of acute IM estimated at 100,000 cases (Evans and Niederman, 1989), EBV causes significantly more illness than mumps, for which a vaccination strategy exists. Another potentially beneficial use of an EBV vaccine is in XLP males, 70% of whom experience an acutely progressive, fatal course of IM during primary EBV infection.

While primary EBV infection stimulates both intense humoral and cell-mediated immunity, each component of this immune response serves a very specific purpose. Vaccines against viruses generally aim to induce humoral immunity against viral structural proteins which neutralize infectious virus, and thereby limit spread to uninfected cells. In contrast, cytotoxic-T-cells recognize and lyse latently infected cells which present processed viral peptides on their cell surface. This latter form of immunity against EBV is particularly important as latently infected cells remain in the peripheral blood for life and serve as a reservoir for viral persistence and/or reactivation to lytic replication and viral shedding. Thus, efforts to design an effective vaccine against EBV should include components capable of inducing humoral and cellular immunity against both lytic and latent gene products.

**Identification of potential vaccine antigens.** Antibody absorption experiments provided the initial evidence that EBV membrane antigens (MA) are responsible for the induction of neutralizing antibodies (Pearson et al., 1971). Similarly, early work on the development of a subunit vaccine against EBV focused on the ability of partially purified MA preparations to induce neutralizing antibody against EBV. The MA complex is comprised of four glycoproteins (gp350, gp250, gp85, gp78) three of which are known to induce neutralizing antibody (Hoffman et al., 1980) (Thorley-Lawson and Geilinger, 1980), and two of which promote ADCC (Pearson et al., 1979) (Khyatti et al., 1991).
EBV glycoproteins present in the membranes of EBV-infected cells

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>EBV Neutralizing Ab</th>
<th>ADCC</th>
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<tbody>
<tr>
<td>gp 350</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>gp 250</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>gp 85</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>gp 78</td>
<td>?</td>
<td>?</td>
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</tbody>
</table>

Adapted from (Wallace et al., 1991).

The most abundant protein on the outer surface of EBV is gp350/220, and most of the human EBV neutralizing IgG antibody response is directed against this glycoprotein (Thorley-Lawson and Poodry, 1982) (North et al., 1982). Accordingly, much emphasis has been placed on producing a gp350/220 based subunit vaccine which will induce neutralizing antibody and ADCC (Thorley-Lawson and Edson, 1979) (Hoffman et al., 1980) (Thorley-Lawson and Geilinger, 1980) (Wallace et al., 1991). Most recently, CD4+ cytotoxic T cell clones have been described which recognize multiple epitopes within the gp350 molecule (Wallace et al., 1991). Additionally, multiple studies have now identified cytotoxic T-cell epitopes in all EBV latent proteins except EBNA-1, and several peptide epitopes have been precisely defined (Khanna et al., 1992) (Murray et al., 1992) (Masucci et al., 1993). The significance of identifying these CTL epitopes is underscored by the recent description of EBV peptide epitopes which sensitize Burkitt's lymphoma cells to lysis by EBV-specific CTL (Khanna et al., 1993).

Studies with gp350-based vaccines. Gp350 is one of the most abundant late viral proteins present in lytically infected cell plasma membranes and the most abundant protein on the outer surface of the virus coat. Importantly, most of the human EBV neutralizing antibody response is directed against gp350 (Thorley-Lawson and Poodry, 1982) (North et al., 1982). Thus, gp350 is the major EBV lytic-cycle gene being pursued in the development of a subunit vaccine. In animal studies, immunization with partially purified gp350 antigen induces EBV-
neutralizing antibody which protects a portion of cotton-top tamarins against a normally lethal, lymphomagenic challenge with EBV (Epstein et al., 1985). Further support for the anti-tumor role played by EBV-neutralizing antibody came from work in which gp350-based immune complexes were used to immunize and effectively protect cotton-top tamarins against EBV challenge (Morgan et al., 1988). In this latter study, protection against EBV-induced lymphoma closely correlated with levels of neutralizing antibody in the serum of immunized animals.

Interestingly, these findings were partially refuted by demonstration that immunization of marmosets with mAb affinity-column purified gp350 did not protect against EBV in spite of high virus-neutralizing titers (Epstein et al., 1986). This finding suggests that in addition to neutralizing antibody, other immune mediators, such as CTL, may be required to prevent the development of EBV-associated lymphomas. In order to overcome the observed variations in immunity induced by various gp350 antigen preparations, the gp350 gene was inserted into a vaccinia virus vector to allow for the homogeneous expression and post-translational processing of this glycoprotein. Curiously, the gp350 vaccinia construct did not induce EBV-neutralizing antibody but did protect cottontop marmosets against a lymphomagenic dose of transforming EBV (Morgan et al., 1988). This unexpected finding clearly suggested the need to examine the cell mediated immune response against this major EBV glycoprotein.

Results of more recent in-vitro experimentation have heightened interest in gp350. A panel of gp350-specific CD4+ T-cell clones from an EBV-immune donor were tested for their proliferative response to a series of truncated gp350 molecules expressed from recombinant DNA vectors in rat GH3 cells, using autologous B-LCLs as antigen presenting cells (Wallace et al., 1991). One T-cell clone recognized a peptide spanning amino acid residues 61-81 of gp350, while three other clones responded to peptides spanning amino acid residues 163-183. An obvious conclusion drawn from this study is the need to screen a variety of gp350-specific T-cell clones
from many EBV-immune donors in order to assess the wider influence of HLA molecule polymorphism upon gp350 epitope choice. This concept has a direct bearing upon the work described in this thesis and our results support the presence of gp350-specific CTL which likely serve to limit virus spread during primary EBV infection.

Clinical implications of EBV vaccines. The identity of the EBV gene products which elicit the aggressive T-cell response during acute IM, and which are responsible for the ability of cytotoxic-T-cells to cause regression of EBV-transformed cells remains unknown and under intense investigation. Along these lines it is important to note that unlike Burkitt's lymphomas which typically express only select EBV latent gene products, the lymphocytes present in post-transplant lymphoproliferative disorders display the same spectrum of latent EBV gene products seen in B cells immortalized in vitro. These findings suggest that development of a vaccine based upon recognition of antigens expressed in EBV-transformed B-cells in vitro will likely confer protection against EBV-induced lymphoproliferative disorders in immunosuppressed patients. Whether an EBV vaccine will even be effective in immunosuppressed individuals remains to be seen. Similarly, vaccine-primed responses against latency type-I infected B cells, expressing EBNA-1, have the potential to promote immunity against phenotypically similar Burkitt's lymphoma cells.

Current understanding of antigen processing and presentation would lead one to predict that the dominant EBV epitopes for cytotoxic T cell recognition will be determined, in part, by an individual's own HLA alleles and will be independent of the original location (membrane, cytoplasm, nucleus, etc.) of the product in the cell. Much remains to be done before the list of EBV epitopes can be considered complete. Using the recombinant vaccinia system described herein, along with access to a diverse panel of acute IM patients and convalescent blood donors, should allow the systematic identification of the immunodominant epitopes of EBV which will be most useful in the rational design of an effective EBV vaccine.
CHAPTER II
MATERIALS AND METHODS

Experimental Design

A major challenge to our understanding of viral latency and immune escape involves a gap in our knowledge regarding the identity of virus encoded proteins which provide epitopes for recognition by virus-specific cytotoxic-T-lymphocytes. In this regard, acute infectious mononucleosis (IM) represents an ideal model system to study virus-specific, cell-mediated immunity. Acute IM is a self-limited illness characterized by the appearance of "atypical" lymphocytes (CD3+/CD8+/HLADR+) which are responsible for virus elimination and provide life-long immunity to Epstein-Barr virus (EBV). The experimental procedures outlined in this section were designed to identify the EBV-encoded latent gene antigens which induce a primary CTL response during acute IM and which maintain EBV-specific memory CTL responses during convalescence.

Lysis of virally infected cells by CD8+ cytotoxic-T-lymphocytes is antigen-specific and MHC class-I restricted, thus all experiments designed to identify EBV antigens were performed using autologous target and effector cells. Blood from acute IM patients and healthy EBV seropositive donors served as a source of peripheral blood lymphocytes to generate bulk CTL cultures and target cells. Lymphocytes were isolated from whole blood by Ficoll-Paque density centrifugation and T- and B-cell enriched populations were obtained by AET-sheep red cell rosette selection. Autologous B cell blasts served as a source of most target cells and individual EBV latent genes were introduced by infection of target cells with recombinant vaccinia virus constructs. Expression of individual EBV gene products in target cells was confirmed by both western blot and immunofluorescence. Primary CTL responses to EBV were evaluated in standard $^{51}$Cr release assays using freshly isolated, T-cell enriched PBMC from acute IM patients as effector cells. Memory CTL responses to EBV were evaluated with bulk CTL cultures...
generated by in-vitro restimulation with autologous B-LCLs. FACS analyses were routinely performed on bulk cultures of effector CTL populations in order to more clearly characterize their phenotype. Lastly, monoclonal antibody blocking studies and cold target competition assays were performed in order to accurately identify the antigenic and MHC components responsible for target cell recognition and lysis.

Patients

The Student Health Services at the University of Massachusetts, Amherst campus, served as the source of acute infectious mononucleosis patients. Students presenting with symptoms consistent with acute IM and having a positive mono-spot test (presence of heterophile antibody) were informed of the study and offered an opportunity to participate. Clinical diagnosis was based upon the presence of fever, swollen tonsillar and cervical lymph nodes, hepatomegaly, splenomegaly and atypical lymphocytes in the peripheral blood. Atypical lymphocytes were enumerated from fresh peripheral blood smears and identified by their large size, eccentric nuclei and abundant irregularly contoured cytoplasm. All patients electing to participate in the study provided written informed consent prior to their enrollment. Serological evaluation of antibody titers to EBV-related antigens was also performed on each patient in order to confirm the diagnosis and characterize the stage of infection as either acute, transitional or convalescent. Additionally, all study participants with a serologically confirmed diagnosis of acute/transitional IM included in this study were born between the years 1970 and 1975 and therefore were not immunized against smallpox (i.e. no previous exposure to vaccinia virus). Five tubes of venous blood (four 15 ml vacutainer tubes containing sodium heparin and one 10 ml tube containing SST gel and clot activator, Becton Dickinson, Rutherford, NJ.) were drawn from each patient on four separate occasions spanning a period of six to eight weeks. The first three acute IM blood samples were obtained within 10-14 days of the first positive heterophile antibody test and during the initial two weeks of symptomatic illness,
these samples were referred to as "acute bloods". The fourth blood samples were drawn approximately 6-8 weeks following the first positive monospot test, and in the majority of cases after all clinical symptoms had subsided, this sample was referred to as the "convalescent blood". Acute bloods served as the source of in vivo-activated cytotoxic-T-lymphocytes (CTL) used to identify EBV-encoded CTL antigens recognized during the primary immune response to EBV infection. In a similar manner, the convalescent blood served as source of memory CTLs used to investigate the specificity of the memory CTL response to EBV. Normal, healthy EBV-seropositive and EBV-seronegative volunteers (laboratory workers) between the ages of 18 and 48 served as the control population. Control samples were collected and handled in an identical manner to the acute IM samples.

**EBV serology.** In order to confirm a diagnosis of acute infectious mononucleosis and define the stage of infection, one ml of serum obtained during the initial patient evaluation was submitted to the clinical serology lab at the University of Massachusetts Medical Center for analysis of EBV-specific antibody titers (anti-EA, anti-VCA IgM, anti-VCA IgG and anti-EBNA). Patients with a serologically confirmed diagnosis of acute IM or acute/transitional status were included in this study.

**EBV strain typing by DNA PCR amplification.** Saliva samples were collected from acute IM patients using an "OraSure" oral specimen collection device (Epitope Inc., Beaverton, OR.) and placed into a 5 ml tube containing 0.5 ml of specimen buffer. PBS was added in the amount required to dilute the original saliva sample by 50%. Virus was precipitated from the diluted saliva sample by addition of 1/2 volume of precipitation buffer: 0.4M NaCl, 30% PEG-8000 (Fisher Scientific, Fair Lawn, NJ.) and the mixture placed at 4°C overnight. Precipitated virus was collected by centrifugation at 800 x g for 30 minutes at 4°C. DNA was prepared (Kawasaki, 1990) by resuspension of the precipitate in 20 μl of proteinase K lysing buffer: 50
mM KCl, 10 mM Tris-Cl pH 8.3, 2.5 mM MgCl₂, 0.5% Tween-20, 0.5% NP-40 and 100 µg/ml proteinase K. Virus was digested at 70°C for 1 hr and the proteinase K was inactivated by heating to 95°C for 10 minutes. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Ten µl of this DNA suspension (equal to 250 µl of saliva) was subjected to PCR amplification.

EBV strain typing by PCR amplification of DNA from 10⁶ unfractionated PBMCs has a highly variable outcome, however, PCR amplification of EBV DNA from 10⁶ SRBC-rosette depleted peripheral blood B cells is routinely diagnostic of the infecting EBV strain. Venous blood served as a source of B cells and was collected (see sample collection and lymphocyte isolation) from acute IM patients at the time of saliva collection. Briefly, an enriched B cell population was obtained by collection of the non-rosette forming fraction of PBMCs subjected to rosette selection with AET-treated sheep red blood cells. DNA was prepared (Kawasaki, 1990) by resuspension of 10⁶ B-enriched cells in 100 µl of proteinase K lysing buffer. Cell suspensions were incubated sequentially at 70°C for 1 hr and 95°C for 10 minutes. Ten µl of this lysate (equal to 10⁵ B-enriched cells) was subjected to PCR amplification. Appropriate controls consist of EBV⁺ and EBV⁻ cell lines including: Ramos; EBV negative B cell line, Namalwa; EBV type-1 Burkitt lymphoma line, and Jijoye; EBV type-2 Burkitt lymphoma line.

The PCR amplification method used to subtype EBV strains is based upon the use of a pair of conserved primers and strain specific probes derived from regions within the EBNA3C gene (Sample et al., 1990). Oligonucleotide primers and probes were synthesized by the Nucleic Acid Facility at UMMC.
5' PCR amplification primer 5' AGAAGGGGACGTGTTGT 3'
3' PCR amplification primer 5' GGCTCGTTTTGACGTCGGC 3'
EBV type-1 specific probe 5' GAAGATTCATCGTCAGTGT 3'
EBV type-2 specific probe 5' CCGTGATTTCTACCGAGAT 3'

PCR amplification was carried out in a 50 μl reaction volume containing 10 μl of DNA sample, 1 μM of each primer, 0.2 μM of each dNTP, 50 mM KCl, 10 mM Tris-Cl pH 8.3, 2.5 mM MgCl₂ and 1 U of Taq DNA polymerase (Perkin Elmer, Norwalk, CT.). Samples were subjected to 35 cycles of amplification in an Ericomp Programmable Cyclic Reactor (Ericomp Inc., San Diego, CA.). Each cycle consisted of: denaturation at 95°C for 1 minute, annealing at 55°C for 1.5 minutes and extension at 72°C for 2 minutes. with a final extension at 72°C for 5 minutes. The amplified products were analyzed by polyacrylamide gel electrophoresis and autoradiography using γ-32P labeled strain-specific probes (Brettler et al., 1992) derived from EBNA3C.

MHC Class I tissue typing. HLA class I tissue typing of acute IM patient PBMCs was performed by the Tissue Typing Facility at the University of Massachusetts Medical Center. Freshly isolated PBMCs, or occasionally, established B lymphoblastoid cell lines (B-LCLs) served as a source of cells for HLA typing. Freshly isolated PBMCs (3x10⁶ cells) were washed in 15 ml of phosphate buffered saline (PBS) and resuspended in 1 ml of RPMI-1640 containing 20% human AB serum (ABS) (Advanced Biotechnologies Inc., Columbia, MD.) and 20 U/ml rIL-2 (Boehringer Mannheim, W. Germany). When B-LCLs or other established cell lines were used for tissue typing, cultures were fed on the day prior to sampling to ensure a viability of >90%. Next, 1x10⁷ cells were washed once in PBS and resuspended in 6 mls of RPMI-1640 containing 10% ABS. Three mls of this cell suspension (5x10⁶ cells) was placed in each of two
polystyrene tubes (Corning Industries., Corning, NY.) and submitted for tissue typing.

Isolation of lymphocytes from peripheral blood

Human peripheral blood from acute IM patients and healthy controls was drawn by veinipuncture into either 50 ml Luer-lok syringes (Becton Dickinson., Franklin Lakes, NJ.) containing 500 units of heparin (10 U/ml whole blood) or alternatively into four 15 ml vacutainer tubes containing sodium heparin (Becton Dickinson., Rutherford, NJ.). Heparinized whole blood was diluted with an equal volume of PBS and 35 ml of the PBS/blood mixture was gently layered onto a 10 ml cushion of 20°C ficoll-Paque (Pharmacia Fine Chemicals., Piscataqay, NJ.) in 50 ml polypropylene tubes. Lymphocyte separation was achieved by centrifugation at 1600 rpm (500 x g) for 30 minutes at 20°C (Maluish and Strong, 1986). Excess supernatant was aspirated from the tubes and the viable mononuclear cell layer was harvested from the Ficoll-PBS interface. PBMCs were washed twice in sterile PBS and cells collected by centrifugation at 1600 rpm (500 x g) for 10 minutes. Washed PBMCs were resuspended at 2x10⁶ cells/ml in RPMI-1640 containing 15% FBS, 2 mM L-Glutamine, 2-mercaptoethanol (2-ME) (Sigma Chemical Co., St. Louis, MO.) 10 μl gentamicin and 0.25 μg/ml fungizone (Gibco BRL, Life Technologies Inc., Grand Island, NY.). Fresh PBMC cultures were grown in a 37°C humidified incubator supplied with 5% CO₂.

SRBC-rosette separation of T- and B-lymphocytes. Sheep red blood cells (SRBCs) (Colorado Serum Co., Denver, CO.) were pretreated with 2-aminopropylisothiouronium bromide (AET) to enhance binding to the CD2 molecule on T cells (Madsen and Johnson, 1979). AET solution was made as follows: 5 gms of AET was dissolved in 100 mls of distilled water and the pH adjusted to 9.0 with 4N NaOH. The final volume was brought to 125 ml with distilled water and the solution sterile filtered through a 0.22 micron disposable filter system (Nalge Company, Rochester, NY.). This volume of AET solution was used to treat 20-40 ml of packed,
washed SRBC or 120 mls of a SRBC suspension as received from Colorado Serum Co. The solution was used only on the same day it was made and excess AET solution was discarded as a several day old solution was observed to lyse the sheep red cells.

Freshly isolated whole PBMC from acute IM patients and normal control donors were mixed with AET-treated SRBCs to form T-cell/SRBC rosettes which were separated into SRBC-rosette positive and negative populations via Ficoll-Paque density centrifugation. Approximately 1x10^7 PBMC in 10 ml of RPMI-1640 were mixed with 3-5 mls of a cold 10% AET-SRBC suspension and centrifuged at 800-1000 rpm (200 x g) for 5 minutes. The supernatant was removed and the cell pellet was placed on ice for 30 minutes. Next, cells were gently resuspended in 10 ml of RPMI-1640 by tilting the tube back and forth and the final volume was brought up to 35 mls with 20°C PBS. The PBMC/SRBC suspension was then layered onto 10 mls of a 20°C Ficoll-Paque cushion in a 50 ml polypropylene tube and centrifuged at 1600-1800 rpm (500-600 x g) for 20 minutes at 20°C. Non-rosette forming cells (mostly B cells) were collected from the Ficoll/PBS interface and washed once in 5-10 volumes of PBS. Rosette forming T-cells were harvested from the cell pellet and washed in 5-10 volumes of PBS. SRBCs present in both the T-enriched and B-enriched pellets were lysed by addition of 5-10 mls of an ice cold NH_4Cl solution: 0.83 gm NH_4Cl/100 ml H_2O, pH adjusted to 7.3 with KHCO_3 and after 1-2 minute incubation, the remaining lymphocytes were washed twice in 5-10 volumes of PBS. Red cell lysis was apparent by the solution becoming dark red and transparent. The cell pellets were resuspended in RPMI-1640 and viable cells counted via trypan blue exclusion. Double- and triple-color fluorescence activated cell sorting (FACS) (Jackson and Warner, 1986) was performed to enumerate T and B cells.

Preparation of target cells

Generation of B cell blasts. B-cell blasts were chosen as targets cells for ^{51}Cr release assays as they are a natural host cell for EBV and are easily generated from fresh or frozen
PBMC (Fothergill et al., 1982) (Khanna et al., 1991). Lymphocytes were isolated from whole blood by Ficoll-Paque density centrifugation and T cells were removed by rosette selection with AET-treated sheep red blood cells as described above. The B cell enriched fraction was then depleted of monocytes by allowing the cells to adhere to plastic for 1 hr at 37°C (Splawski and Lipsky, 1991). The non-adherent B cells were collected and stained with a panel of fluorochrome-conjugated monoclonal antibodies for surface marker phenotype analysis. The enriched B cell population served as a source for generating anti-IgM-stimulated B-cell blasts and for establishing EBV-transformed B-lymphoblastoid cell lines (B-LCL).

The enriched B lymphocytes were cultured at a concentration of 1-2x10^6 cells/ml in RPMI-1640 containing 10-15% ABS, highly purified human recombinant 20 U/ml rIL-2, 50-100 U/ml rIL-4 (Genzyme, Cambridge, MA.), 1-10μg beads/10^6 cells rabbit anti-human IgM (μ-chain specific) coated acrylamide beads (Irvine Scientific, Santa Ana, CA.). Following culture for 48-72 hr, the B-cell blasts served as suitable targets for infection with vaccinia virus constructs. The ideal conditions for generating B-cell blasts (1μg IgM-beads/10^6 cells, 100U/ml rHu-IL-4 and 20U/ml rHu-IL-2) were confirmed by FACS analysis.

**Generation of B-lymphoblastoid cell lines (B-LCLs).** All B-LCLs were established by infecting peripheral blood mononuclear cells (PBMC) with EBV-containing culture supernatants from the EBV type-1 producer cell line; B95-8 (Nilsson et al., 1971) (Tosato, 1991). Unfractionated PBMC served as a source of resting B cells. Unstimulated B cells and not B cell blasts were used here as EBV infects resting B cells in preference to activated B lymphocytes (Aman et al., 1984). B95-8 supernatant was produced by inoculating 100 ml of RPMI-1640 with 1x10^6 cells/ml exponentially growing B95-8. The culture was incubated in a humidified 37°C, 5% CO₂ incubator for 5-10 days without additional feeding. The EBV-containing culture supernatant was harvested by centrifugation for 10 minutes at 1600 rpm (500xg). The supernatant was then passed through a 0.45-μm filter and 1 ml aliquots containing >10^2-10^3
transforming units/ml were stored frozen at -80°C. New B-LCL lines were initiated by infecting 10^7 unfractionated PBMC or 2x10^6 enriched B cells in 2-3 ml of RPMI-1640 with 0.5 ml of B95-8 supernatant stock. Newly infected cultures were incubated at 37°C for 2-3 hr and fed with 5 ml of RPMI-1640 containing 15% FBS. Memory CTL from EBV-seropositive individuals typically prevent the outgrowth of EBV immortalized B cells in vitro and were usually either removed by SRBC-rosetting or functionally inactivated with cyclosporin A (Tosato et al., 1982) (Bejarano et al., 1985) (Rickinson et al., 1979). When unfractionated PBMC were used, cyclosporin A (0.1μg/ml) was added to the media during the first several weeks after initiating the culture (Rickinson et al., 1984). Newly established cultures were carefully observed for the appearance of proliferating clumps of cells, characteristic of EBV-transformed B cells. Proliferating cultures were expanded by twice weekly feedings with RPMI-1640 containing 10% FBS and cell density was maintained at 1-2x10^6 cells/ml. A complete list of established B-LCLs used in this work and their MHC class-I haplotype is shown in Table 2. For long-term storage, aliquots of each line were frozen in RPMI-1640 containing 10% FBS and 10% DMSO.

**Generation of HTLV-1-transformed T-cell lines.** HTLV-1-transformed T-cell lines were generated by co-cultivation of PBMC with the HTLV-1 secreting MT-2 cell line as described previously (Merl et al., 1984) (Nutman, 1991). Briefly, PBMCs were isolated from heparinized whole blood by Ficoll-Paque density centrifugation. Phytohemagglutinin (PHA)-blasts were generated by placing unfractionated PBMC at 10^6 cells/ml in RPMI-1640 containing 0.5mg/ml PHA (Burroughs Wellcome., Research Triangle Park, NC.) and incubating at 37°C for 72 hrs. As a source of HTLV-1, one half as many mitomycin-C treated (50μg/ml for 1 hr at 37°C) MT-2 cells were added to the PHA-stimulated T cells. Growth of the transformed T cells was maintained by addition of 10-20 U/ml rIL-2 to the media. These T cell lines remained IL-2 dependent and were typically >95% CD4 positive.
Generation of primary human fibroblast lines. As a source of human dermal fibroblasts, 3-4mm skin biopsies were obtained from healthy donors. Tissue samples were placed in 100 mm² tissue culture plates and 1-2 ml of a 10x trypsin solution: 2.5 mg trypsin/ml in Tris-NaCl pH 7.5, Gibco BRL, Grand Island, NY., was added. The tissue was teased apart using two sterile scalpels and subsequently examined under the microscope to ensure that the fibroblasts had been freed from remaining clumps of tissue. The trypsin/cell suspension was diluted in 10 ml of PBS and the fibroblasts were collected by centrifugation at 1500 rpm (400 x g) for 5 minutes. The supernatant was removed and the cells were resuspended in 1-2 ml of growth medium: RPMI-1640 containing 20% FBS, 4mM L-glutamine, 1mM Na-Pyruvate, 0.1mM non-essential amino acids, 30μg/ml heparin, 25 ng/ml of each of acidic and basic recombinant human fibroblast growth factor (Sigma Chemical Co.,) 60μM 2-mercaptoethanol and 10μg/ml gentamicin. Cells from each biopsy specimen were then placed into 3-4 wells of a 24 well plate and grown in a 37°C, 5% CO₂ humidified incubator. One to two weeks of incubation time were typically required before substantial growth was observed. Successfully established lines were allowed to grow until cells covered the bottom of a tissue culture flask and the monolayers were harvested and expanded by preparing a single cell suspension using a 1x trypsin solution: 0.25 mg trypsin/ml PBS. Excess fibroblasts were frozen in RPMI-1640 plus 10% DMSO and stored in liquid nitrogen until needed.

Growth of vaccinia virus stocks

The recombinant vaccinia vectors encoding single EBV genes utilized in this work were kindly provided by Dr. Elliot Kieff. Table 3 lists the recombinant vaccinia vectors along with names and sizes of the inserted EBV open-reading frames. Details of the vaccinia vector construction can be found in (Murray et al., 1990) (Khanna et al., 1992).
Growth of vaccinia virus crude stocks. CV-1 cells were grown to 90% confluency in Minimal Essential Medium (MEM) plus 10% FBS, and washed in PBS prior to infection in order to enhance virus absorption. High titer vaccinia virus stocks were diluted to $10^6$-$10^7$ pfu/ml in plain MEM and 5 ml were added to each 150 cm$^2$ flask of CV-1 cells. Virus was allowed to absorb for two hours in a 37°C incubator with occasional rocking. Infected monolayers were fed with 30-40 ml of fresh MEM containing 5% human AB serum (ABS) and allowed to incubate for 48-72 hours. During this incubation the cytopathic effect (CPE) of the virus was apparent as the monolayer of adherent cells round-up and sloughed from the surface of the flask. Virus stocks were prepared from these mature cultures by several freeze/thaw cycles when CPE was evident in 80-90% of the CV-1 cells. The mature cultures were frozen at -20°C over night, partially thawed at room temperature and the slurry shaken vigorously several times in order to scrape all cells free into the media. The cell debris was collected in a low speed centrifuge for at 1200 rpm for 10 minutes and the pellets resuspended in 2 ml of MEM. To release cell-associated virus, the cell suspensions were subjected to three quick freeze/thaw cycles by alternate submersion in a dry ice-ethanol bath and a 37°C water bath. Additional virus was released from the cell pellet by treatment with trypsin. Fifty μl of 10x tissue culture trypsin solution was added to each 2 ml cell suspension and incubated at 37°C for 30-45 minutes. The crude virus stock was dispensed in 100 μl aliquots and frozen at -80°C with titers typically in the range of $10^8$ pfu/ml. This virus was used only to seed new stock cultures. Purified virus stocks used to infect target cells were prepared by sucrose gradient centrifugation of the crude stocks.

Sucrose gradient purification of vaccinia virus stocks. Preparation of purified vaccinia virus stocks required more starting material than for crude virus stocks, typically five or more 150 cm$^2$ flasks of CV-1 cells were infected with each vaccinia construct. The cell pellets obtained from the monolayers undergoing CPE were resuspended in 1mM Tris-HCl pH 9.0
instead of MEM. The cell suspensions were subjected to several rounds of freeze/thawing and treatment with trypsin as was done in preparing the crude stocks. Virus was then separated from cell debris by high speed centrifugation through a sucrose cushion. A 10 ml cushion of 36% sucrose made in 1mM Tris-HCL pH 9.0 was placed in the bottom of each Oak Ridge centrifuge tube and the virus preparations, in a final volume of 20 ml, were laid on top. The tubes were centrifuged at 20,000 x g for 80 minutes and the supernatant removed by aspiration. Virus pellets were resuspended in 2 ml of 1mM Tris-HCl pH 9.0 with resulting titers in the range of 2 x 10^9 pfu/ml. The sucrose gradient purified virus stocks were used for infection of target cells.

**Titration of vaccinia virus stocks.** Virus stocks were titered via a plaque forming assay on monolayers of adherent cells. CV-1 cells were grown to confluency in 6 well plates and used as the indicator cell line. CV-1 monolayers were washed with MEM and 1 ml of various dilutions (10^-3 to 10^-7) of virus were added in duplicate to the wells. The virus was allowed to absorb for 2 hrs at room temperature with periodic shaking. The inoculum was then removed and replaced with 4 ml of a 40°C mixture containing equal parts of 2x plaque assay media: MEM plus 5% ABS and 2% Seaplaque agarose (Sigma Chemical Co.). The agar was allowed to set at room temperature for 15 minutes and the plates incubated at 37°C for 48 hrs. Culture wells were then overlayed with 2 ml of X-gal solution (Sigma Chemical Co.) containing 500 mg X-gal/ml in 1% Seaplaque. Plates were developed overnight in a 37°C incubator and plaques in duplicate wells were counted and averaged to determine viral titers.

**Expression of recombinant vaccinia-EBV constructs in target cells**

Primary human fibroblast lines, HTLV-1 transformed human T cell lines and anti-IgM stimulated B-cell blasts were infected with recombinant vaccinia virus constructs at a multiplicity of infection (MOI) of 5:1 to 10:1 at 37°C for 1 hour. Following infection, cells were incubated for 4-24 hours to allow for expression of the vaccinia-encoded EBV latent gene
product. At four hour intervals portions of the infected cells were harvested for either western blot or immunofluorescence analysis. EBV-encoded proteins were detected with either polyclonal human serum from EBV seropositive donors or when available, a mAb against the specific EBV protein.

**Immunofluorescent detection of EBV nuclear antigens.** Fibroblast lines grown to near confluence in MEM plus 15% FBS were harvested by trypsinization and plated at low density onto 8-chamber tissue culture slides (Nunc, Inc. Naperville, IL.). Fibroblasts were again grown to near confluence at 37°C in a humidified 5% CO₂ incubator, washed in 0.2M PBS pH 7.2 and infected with vaccinia constructs at an MOI of 10:1. Virus was allowed to adsorb to the fibroblast monolayer for 1-2 hrs at 37°C. Excess virus was removed by washing individual wells with PBS and the infected cells were fed with fresh MEM and allowed to grow for 4-24 hrs. Prior to immunofluorescent staining, all cells were first washed 3 times in Hank's Balanced Salt Solution (HBSS) without Mg²⁺ and Ca²⁺. Slide preparations of non-adherent cells (K562 and B-LCLs) were made by placing 10-20 µl of a cell suspension containing 2-5 x 10⁴ cells in each well of an 8-well slide. Slides were air dried overnight, fixed in a 4°C mixture of methanol/acetone (50% v/v) for 15 minutes and washed several times in PBS containing 1% FCS. Prior to specific staining, all cells were permeabilized with 0.5% NP40 in PBS plus 1% FCS for five minutes at 20°C and washed once more in PBS. Donor serum from EBV seropositive/vaccinia seronegative individuals served as a source of EBNA-specific primary antibody. All donor serum was heat inactivated at 56°C for 30 minutes prior to use in order to inactivate complement. Complement free serum was diluted 1/100-1/500 in HBSS and 10-20 µl was added to each well and allowed to incubate for 30 minutes in a humidified 37°C incubator. The slides were then washed by soaking twice for 5 minutes each in HBSS with low speed stirring. Washed slides were rinsed in distilled water and 15-20 µl of a 1/10 dilution of EBV negative, vaccinia negative human serum was added to each well. This EBV⁺/Vac⁺ serum
served as a source of human complement. Slides were then incubated at 37°C in a moist chamber for 30 minutes followed by several washes in PBS and rinsed in distilled water. Detection of specific immune complexes was achieved by addition of 15-20 µl of a 1:100 dilution of goat anti-human C3 FITC-conjugated mAb. Slides were incubated with the FITC-conjugated mAb for 30 min at 37°C in a moist chamber and again washed as described above. Coverslips were applied to the air dried samples using a solution of glycerin/Hank’s BSS (1:1). Slides were examined and photographed at 400-1000 X with a Zeiss Axioplan fluorescent microscope (Zeiss Inc. West Germany).

**Immunofluorescent detection of EBV latent membrane protein 1.** The LMP1-specific mAb S12 was provided by Dr. Elliot Kieff. Cell samples were prepared in 8-well chamber slides as described above for the detection of EBNAs (Winchester and Ross, 1986). To block nonspecific binding of antibodies to the cell membrane, the fixed cells were first incubated with 2% goat serum diluted in HBSS for 30-60 minutes at 37°C. Slides were washed by sequential submersion in HBSS and distilled water for 5 minutes each, without allowing slides to dry completely between the various steps. As a primary reagent, the LMP-1-specific, murine mAb S12 was added to each well at a 1/500 dilution and allowed to incubate for 1-2 hrs at 37°C. Following this incubation, the slides were again washed in HBSS and distilled water. As a secondary/detecting reagent, FITC-conjugated goat anti-mouse Ig was added at a 1:20 dilution and incubated at 37°C for 30 minutes. The slides were washed a final time, dried completely and a coverslip placed over the sample using a solution of glycerin/Hank’s BSS (1:1). Cells were photographed at 400-1000 X using a Zeiss Axioplan fluorescence microscope (Zeiss Inc. West Germany).

**Immunochemical detection of EBV gp350/220.** This procedure was carried out in a cell suspension at 4°C and unlike the staining protocols for LMP-1 and the EBNAs, these cells were
not fixed prior to staining (Winchester and Ross, 1986). Approximately 1x10^6 cells were harvested from a vigorously growing culture and washed several times in cold PBS. Cells were first treated with a 1/100 dilution of a murine anti-gp350/220 mAb 72A1, and incubated at 4°C for 30 minutes. The cells were then washed in ice cold PBS to prevent capping of the antibody on the cell surface. As a secondary reagent, the cells were treated with a 1/20 dilution of FITC-conjugated goat anti-mouse Ig and again allowed to incubate for 30 minutes at 4°C. Excess FITC-conjugated Ab was removed by three washes in cold PBS and the cells were resuspend in 50-100 μl of this same solution. Ten μl of the stained cell suspension was placed on a slide and covered with a coverslip. The slides were examined as above.

**Immunoblot detection of EBV nuclear antigens.** Expression of vaccinia encoded EBV genes was evaluated in a variety of human cell lines including primary human fibroblast lines, HTLV-1 transformed T cell lines and anti-IgM stimulated B cell blasts (Garfin, 1992). Recombinant vaccinia virus constructs were added to the cell suspensions at an MOI of 10:1 and incubated at 37°C for 1-2 hr to allow for virus adsorption. Total cell lysates were prepared by harvesting sequential fractions of the infected cultures at 4, 8, 12, 16 and 24 hrs. Briefly, 2-5x10^6 cells were collected via centrifugation and the volume of the cell pellet was estimated. Approximately ten volumes of sample buffer was added to the cell pellet and mixed vigorously. The sample buffer contained 2% SDS, 100 mM dithiothreitol, 60 mM Tris pH 6.8 and 0.01% bromophenol blue. Samples were boiled for 3-5 minutes and chromosomal DNA was sheared by repeated passage through a 20-gauge and subsequently a 26-gauge needle. The samples were then centrifuged at 10,000 x g for 10 minutes, supernatant recovered and any insoluble pellet discarded.

The protein samples prepared in this manner were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose paper via semi-dry electrophoretic transfer. The transfer buffer contained 190 mM glycine, 25 mM Tris-base pH 8.0 and 20%
methanol. In general, proteins were transferred from the 6x8 cm gels for 2-4 hrs at 50mA/gel. The nitrocellulose blots were washed several times in PBS prior to blocking non-specific binding sites with a Blotto/Tween solution: 5% non-fat dry milk, 0.2% Tween-20 and 0.02% sodium azide in PBS for 1-2 hrs at room temperature. The blots were washed twice for 5 minutes each in PBS + 0.1% Tween. Serum from EBV seropositive donors was used as a source of primary antibody which was diluted 1/100 - 1/500 in Blotto/Tween and allowed to incubate with the blot for 1-2 hr at 20\(^\circ\)C. Longer incubations typically increased sensitivity. Blots were washed in 2 changes of PBS + 0.1% Tween-20 wash buffer for 5 minutes each, followed by two more washes in alkaline phosphatase wash buffer. The alkaline phosphatase wash buffer contained 150mM NaCl, 50 mM Tris-base pH 7.5 and 0.1% Tween-20. Alkaline phosphatase conjugated goat anti-human IgG (Tago, Inc., Burlingame, CA.) was used as the secondary/detection antibody which was diluted to a concentration of 1-2mg/ml in alkaline phosphatase wash buffer + 5% non-fat dry milk. Blots were incubated with the secondary Ab for 1-2 hr at room temperature with agitation and the excess antibody-conjugate thoroughly removed by washing the blot several times in alkaline phosphatase wash buffer. Blots were developed by incubation in a solution containing the substrates for the alkaline phosphatase enzyme, bromochloroindoyl phosphate and nitro blue tetrazolium (BCIP/NBT) (Kirkegaard & Perry Labs Inc. Gaithersburg, MD.). The washed blots were placed in substrate solution and allowed to develop at room temperature with agitation until bands were sufficiently dark. Development typically took about 5-10 minutes and was stopped by rinsing the blots in PBS containing 20 mM EDTA.

**Immunoblot detection of EBV latent membrane protein 1.** As described above for the immunoblot detection of EBNAs (Garfin, 1992) The LMP1-specific murine mAb S12 was used as a primary reagent, kindly provided by Dr. Elliot Kieff. Alkaline phosphatase-conjugated goat-anti-mouse antibody was used as the secondary reagent (Tago Inc. Burlingame, CA.).
Preparation of effector cells

Various T lymphocyte-enriched populations were used as effector cells in $^{51}$Cr release assays which differed primarily in whether or not the CTL were restimulated in-vitro, and by the source of antigen used for restimulation. Whole PBMC preparations from acute IM patients and healthy controls routinely contain significant numbers of CD16$^+$ natural killer (NK) cells, which were removed by AET-SRBC selection of T cells and monoclonal antibody (mAb) directed complement mediated lysis of NK cells.

**Natural killer cell depletions.** All whole PBMC preparations and T cell-enriched populations which served as effector cells in $^{51}$Cr release assays were specifically depleted of NK cells by mAb directed complement mediated lysis (Kanof, 1991). The cells were thoroughly washed in PBS, collected by low speed centrifugation and resuspended at $5 \times 10^7$ cells/ml in $4^\circ$C RPMI-1640 containing 0.3% BSA. Media containing FBS was avoided as it significantly increased nonspecific cell lysis. MAb Leu11b (anti-CD16, 0.05 $\mu$g/10$^6$ cells, Becton Dickinson, San Jose, CA.) was added and the cell suspension and incubated for 30-45 minutes. Cells were washed twice in 25 volumes of ice cold PBS and resuspended in 2 mls of cold RPMI-1640 plus 0.3% BSA. Lyophilized rabbit serum (Accurate Chemical and Scientific Corp., Westbury, NY.) which served as the source of complement, was resuspended in 5-6 ml of RPMI-1640 plus 0.3% BSA and filter sterilized by passage through a 0.45$\mu$m Acrodisc PF filter (Gelman Sciences., Ann Arbor, MI.). The sterile complement suspension was added to cells at a final concentration of $2 \times 10^7$ cells/ml and the mixture was then incubated for 45-60 minutes in a $37^\circ$C shaking water bath. The complement treated cells were washed in PBS, collected via centrifugation and resuspended at $1 \times 10^6$ cells/ml in RPMI-1640 supplemented with 15% FBS and 10$\mu$g/ml gentamicin.
Generation of EBV-specific memory CTL cultures. Previous studies on the cell-mediated immune response to Epstein-Barr virus conducted in our lab demonstrated that lysis of EBV infected cells was primarily mediated by CD3+/CD8+, HLA Class-I-restricted T cells (Tomkinson et al., 1987). Thus, this procedure describes the in-vitro expansion of a population of activated, EBV-antigen-specific CTLs. Freshly isolated PBMC from healthy EBV seropositive donors were used as a source of virus-specific, memory T-cells. Lymphocytes were separated from whole blood by Ficoll-Paque density centrifugation as described earlier. Bulk T cell cultures were initiated with an enriched T cell fraction obtained by one round of SRBC-rosette selection, followed by depletion of NK cells with mAb Leu11b plus complement.

Cultures were initiated by stimulating 5x10⁷ enriched T cells (responder cells) with 1.25x10⁶ mitomycin-C-treated (50 μg/ml) autologous BLCLs (stimulator cells) (Strang and Rickinson, 1987) (Wallace et al., 1982) (Sugamura et al., 1981) (Sugamura and Hinuma, 1980).

This initial stimulation was always carried out at a responder to stimulator ratio (R:S) of 40:1. The high R:S ratio provides optimum conditions for generating an antigen-specific T-cell-mediated response (Wallace et al., 1982) by minimizing excess antigen and eliminating the non-specific stimulation of either cross-reactive or lower affinity T cell receptors (Svedmyr et al., 1974). Responder cells were then diluted to a concentration of 10⁶ cells/ml in RPMI-1640 containing 15% FBS, 2mM L-Glutamine, 50μm 2-mercaptoethanol, 10μg/ml gentamicin, 0.25μg/ml fungizone. Two ml of this cell suspension were then added to each well of a 24 well plate and incubated in a 37°C humidified incubator supplied with 5% CO2 for 5-6 days. Next, one half of the media was replaced with fresh RPMI-1640 containing 15% FBS, 20U/ml rIL-2, 2mLM-Glutamine and 50μM 2-mercaptoethanol. On day 8-10, half of the media was replaced with fresh media containing 40U/ml rIL-2 (20U/ml final) and the culture incubated for an additional 2-4 days. On day 10-14 after initiating the culture, responder cells were re-stimulated with mitomycin C treated autologous B-LCLs at a responder to stimulator (R:S) ratio of 3:1 and fed with fresh RPMI-1640 containing 20U/ml rIL-2. Beyond this point cultures
were maintained with twice weekly feedings and restimulated weekly with autologous B-LCLs at a R:S ratio of 3:1.

Target cell specificity of the responder cells was tested after the culture had been maintained for at least 18 days, typically after three or more stimulations with B-LCLs. Responder cells were harvested and tested for their ability to lyse a variety of target cells as measured in a standard $^{51}$Cr release assay. Target cells consisted of both EBV positive- and EBV negative-cell lines as well as MHC class-I matched (autologous) and MHC class-I mismatched (allogeneic) cells.

Cytotoxic T lymphocytes from acute IM patients. Several independent groups of investigators have described the high levels of apoptosis observed in cultures of activated CTLs (CD8+/HLA-DR+) from acute IM patients (Uehara et al., 1992), (Moss et al., 1985). These highly sensitive cells can be rescued from apoptotic cell death by in-vitro culture in the presence of exogenously added IL-2 (Akbar et al., 1993), (Cohen, 1991). The initial steps involved in preparation of cytotoxic T cells from acute IM patients was similar to that of convalescent donors described above. PBMC were first enriched in T cells via one round of SRBC-rosetting followed by mAb Leu11b/complement depletion of NK cells. T cell enriched populations were grown at $10^6$ cells/ml in RPMI-1640 supplemented with 15% ABS, 20U/ml rIL-2, 50µM 2-mercaptoethanol and 2mM L-Glutamine. The enriched T cell cultures were typically maintained for 48-72 hrs without restimulation, in a 37°C humidified atmosphere containing 5% CO$_2$. The virus-specific lytic activity of this T-enriched culture was readily detected on day one of culture and could be maintained for up to 2 weeks without restimulation. Virus-specific lytic activity could also be maintained for up to 1-2 months by periodic restimulation with an empirically determined dilution of OKT3 ascites, as a source of anti-CD3 mAb, and rIL-2. The surface marker phenotype of CTLs prepared in this manner were routinely 95-98% CD3$^+$, 60-80% CD8$^+$ and 20-40% CD4$^+$. 
Recombinant vaccinia-EBNA1 stimulated bulk CTL cultures. This method was devised to selectively stimulate and expand EBV-specific class-I restricted CTLs which specifically recognized an as yet unidentified epitope within the EBNA 1 protein. The source of PBMCs for these experiments came from a diverse panel of donors having varying characteristics with respect to stage of EBV infection, vaccinia immunization status and antigen specificity of their memory CTL response to EBV. PBMCs from various donors were handled differently depending upon the donors' stage of EBV infection, acute vs convalescent. Activated T cells isolated from acute IM patients undergo apoptotic cell death if deprived of IL-2 and therefore these cells were supplemented with rIL-2 at the time of initiating the cultures. In contrast, bulk CTL cultures derived from convalescent donors are not prone to apoptosis and were therefore deprived of IL-2 until the 5th-6th day of culture. In order to expand an antigen specific population of CTLs in vitro, best results were achieved when the responding cells were stimulated with antigen and initially deprived of exogenous IL-2. This procedure favors the survival of only those T cells specifically stimulated by the antigen of interest. Antigen specific T cells activated by the initial stimulation readily expand when supplied with IL-2 on day 5-6. Stimulator cells consisted of autologous B cell blasts or B-LCLs infected with the vaccinia-EBNA1 construct and fixed in aldehyde. The stimulator cells were infected with the vaccinia-EBNA1 construct at an MOI of 5, incubated at 37°C for 1 hr, fed with fresh RPMI and then grown for 12-16 hrs so as to allow for expression of the EBNA1 protein. Vac-EBNA1 expressing cells were then subjected to aldehyde fixation, which inactivated the vaccinia virus, prior to the use of these cells as a source of antigen. The chemical reactivity of P-formaldehyde with proteins is well established and involves reductive methylation and cross-linking of lysine residues by reaction with the free ε-amino groups (Pancake and Nathanson, 1973). In the work described here various methods of aldehyde fixation were evaluated in an effort to minimize any modification of the native EBNA1-MHC antigenic structure and/or
costimulatory molecules on the antigen presenting cell. Formaldehyde fixation of cells (Maccario et al., 1992) consisted of resuspending the cells in a 0.5% solution of formaldehyde for 10 minutes followed by several washes in PBS. However, an alternative, more gentle glutaraldehyde fixation method was utilized for the majority of experiments described here (Hosken et al., 1989) (Hapel et al., 1980). Following 12-16 hrs of vaccinia infection, cells were incubated in 50μg/ml mitomycin-C for 1 hr at 37°C, washed twice in PBS and resuspended in 1 ml of plain RPMI. Freshly prepared 2% glutaraldehyde (Grade I, Sigma Chemical Corp.) was added to a final concentration of 0.05% and the cells vortexed gently for 15 seconds. Aldehyde fixation was stopped by addition of an equal volume of 0.2M L-Lysine (Sigma Chemical Corp.) followed by a one minute incubation. The fixed cells were washed twice in 25 volumes of PBS and used as stimulator cells. In all cases PBMC cultures were stimulated at an initial R:S ratio of 40:1 with subsequent stimulations at a R:S ratio of 3:1 once weekly thereafter. Beyond the 5th-6th day of incubation, all cultures were maintained in the presence of 20U/ml rIL-2. Target cells for ⁵¹Cr release assays included unfixed stimulator cells among the panel.

The following figure is a schematic summary of the various stimulation procedures used to generate the effector cells outlined in this section.
Generation of Virus-Specific CTL

**Primary stimulation in-vivo**

**Stimulator Cell**
- BLCL (In-vivo)

**Responder Cell**
- Naive CD8+ T cell
- HLADR- CD45RA+

**Activated CD8+/HLADR+ T cell**

- Activated CTL present during Acute IM

**Secondary stimulation in-vitro**

**Stimulator Cell**
- BLCL grown in-vitro

**Stimulator Cell**
- B cell blast + Vac-EBNA1

**CD8+/HLADR-CD45RO+ Memory T-cell**

**Identify antigens recognized by EBV-specific CTL**

**Identify antigens recognized by EBV-specific CTL**

**B-LCL expanded CD8+ CTL**

**EBNA1 expanded CD8+ CTL**

**Identify antigens recognized by EBV-specific CTL**

**Identify antigens recognized by EBV-specific CTL**
Chromium release assays

Infection of target cells with vaccinia-EBV constructs. Recombinant vaccinia virus constructs encoding single EBV genes were used to introduce EBV proteins into non-EBV infected cell lines in order to generate targets for $^{51}$Cr-release cytotoxicity assays (Khanna et al., 1991). Actively dividing cells serve best as targets for infection with vaccinia constructs as they are readily infected and promote high levels of vaccinia driven gene expression. Target cells were always grown in media supplemented with human ABS and not FBS. T- and B-cell blasts were harvested from actively dividing cultures, washed thoroughly in PBS and collected by centrifugation for 5 minutes at 1000-1200 rpm in a Beckman GPR centrifuge using a GH-3.7 rotor. The viability of uninfected target cells was always >90% by trypan blue dye exclusion. In order to increase viability, collection of cells on a Ficoll-Paque cushion was sometimes necessary. The target cell pellets were resuspended in RPMI-1640 supplemented with 5% ABS at a concentration of $5 \times 10^6$ cells/ml. A volume of approximately 100-200 µl of this cell suspension (0.5-1x$10^6$ cells) was needed for each vaccinia construct to be tested. Vaccinia virus constructs were added to target cells at an MOI of 5:1 (50 µl of a 1x$10^9$ titer stock) and incubated at 37°C for 1-2 hr to allow for virus adsorption. The infected cells were washed in PBS to remove any excess virus, fed with several mls of RPMI-1640 containing 5% ABS and incubated at 37°C for 12-16 hrs. Target cells were collected via centrifugation at 1000-1200 rpm for 3-5 minutes and all but 200 µl of the supernatant was removed by aspiration. The cells were then radiolabeled by addition of 100 µCi of $^{51}$Cr-Na$_2$CrO$_4$ at 100 µCi/target (Dupont N.E.N., Boston, MA.) and incubated at 37°C in a shaking water bath for 1-2 hrs. Radiolabeled cells were then washed twice in 5 ml of PBS and collected by centrifugation at 1200 rpm for 3-5 min. The washed cells were resuspend at $1 \times 10^5$/ml in RPMI-1640 supplemented with 5% ABS and 100 µl (1x$10^4$ cells) was placed in each test well of a 96 well round bottom microtiter plate.
Chromium release assays. The cytotoxic activity of the various lymphocyte effector populations was evaluated in a standard $^{51}$Cr-release assay (Whiteside et al., 1992). The assays were carried out in round bottom 96 well microtiter plates containing $1 \times 10^4$ $^{51}$Cr-labeled target cells seeded into each well. Approximately $0.5-1 \times 10^6$ cells were infected for each vaccinia construct to be tested. Targets were infected with recombinant vaccinia constructs at an MOI = 5:1 to 10:1 and incubated for 16-20 hrs at $37^\circ$C to allow for sufficient expression of the introduced EBV gene. Target cells ($0.5-1 \times 10^6$) were $^{51}$Cr radiolabeled by incubation with 100 µCi of $^{51}$Cr for 1-2 hr at $37^\circ$C. Radiolabeled targets were washed twice in PBS, resuspended at a concentration of $1 \times 10^5$ cells/ml in RPMI containing 10% ABS and 100 µl ($1 \times 10^4$ cells) added to each well of a 96 well round-bottom plate. All effector/target combinations, including maximum and spontaneous release wells, were set-up in triplicate. Typically, effector cells were added to wells containing targets at various ratios ranging from 0.5:1 to 50:1 ($5 \times 10^3$ - $5 \times 10^5$ effector cells/well). The actual number of vaccinia constructs tested and the effector:target (E:T) ratios used for each assay varied slightly from patient to patient depending upon the number of effector and target cells available. The assay plates containing effectors and targets were centrifuged briefly at 800 x g, incubated for 4-6 hrs at $37^\circ$C, recentrifuged and 100 µl of supernatant harvested from each well for counting in a γ-counter (Beckman Instruments, model 5500B). Spontaneous release of $^{51}$Cr was quantitated by incubating target cells in media alone, in the absence of effector cells. Maximum release of radioactivity was determined by quantitating the radioactivity in a 50 µl aliquot of the original target cell suspension. Percent specific $^{51}$Cr release was calculated as follows: % specific release = $100 \times \frac{[(\text{sample cpm}) - (\text{spontaneous cpm})]}{[(\text{maximum cpm}) - (\text{spontaneous cpm})]}$.

Spontaneous $^{51}$Cr release did not exceed 25% of the maximal incorporation for any of the data shown. Criteria for significant lysis were that exceeding background lysis by at least 10%. More typically, specific lysis exceeded background lysis by >25%.
**Inhibition of CTL-mediated killing by monoclonal antibody.** Monoclonal antibody blocking studies were undertaken to define the cellular phenotype of the cells mediating lysis. Inhibition of cytotoxic activity was achieved by adding various dilutions of mAb stock solution (ascites or hybridoma culture supernatant) to $1\times10^4$ $^{51}$Cr labeled targets in 100 µl of media for 30 minutes, after which various numbers of effector cells in 100 µl of media were added. The inhibitory effect of the mAb on cytolysis was assessed following a 4-6 hour $^{51}$Cr release assay as previously described. Inhibition was calculated as: 

\[
\% \text{ inhibition} = 100 \times \left( \frac{\text{cytotoxic activity of effector cells in the absence of mAb} - \text{cytotoxic activity of effector cells in the presence of mAb}}{\text{cytotoxic activity of effector cells in the absence of mAb}} \right)
\]

**Cold target competition assay.** In effort to identify the MHC class-I restriction molecule and viral protein specifically involved in antigen recognition, cold target inhibition studies were performed. Cells used as cold target competitors consisted of a variety of EBV or Vac-infected and uninfected, unlabeled “cold” target cells, which were added at 10 fold excess to compete with radiolabeled targets in a standard $^{51}$Cr release assay. Cold targets sharing MHC/peptide antigen complexes with “hot” targets compete as targets for antigen-specific lysis and thereby reduce lysis of radiolabeled target cells. Target cells were labeled with $^{51}$Cr as described previously and $1\times10^4$ hot targets in 50 µl of RPMI were added to each well of a 96-well plate. To competitor wells, $1\times10^5$ cold competitors in 50 µl of media was also added. Fifty µl of media was added in place of competitor cells to wells used to measure specific release. Various numbers of effector cells in 100 µl of RPMI were added to all specific release wells and cold competitor wells. For maximum release wells, 150 µl of media was added to the hot targets in place of effector cells and 100 µl of this cell suspension was harvested for analysis in a γ-counter. Wells used to measure spontaneous release were set-up in a similar manner to the maximum release wells, except that only the cell free supernatant was harvested for counting. Effector cells ($5\times10^5$ cells in 100µl) were added to all wells except the maximum and
spontaneous release wells. The final volume per well was 200 μl in all cases.

Cytofluorographic analysis

Double and triple color cytofluorographic analysis was performed on a fluorescence activated cell sorter (FACSScan, Becton Dickinson, Mountain View, CA.). Cell staining was accomplished via the direct method using antibodies conjugated to either fluorescein isothiocyanate (FITC), phycoerythrin (PE) or percp (PCP) (Jackson and Warner, 1986). Prior to staining, all cells were washed in ice cold PBS containing 0.2% sodium azide (PBS/azide) and resuspended at 5x10^7/ml in cold PBS/azide. For each set of surface markers to be evaluated, approximately 1x10^6 cells (20μl of the cell suspension) were incubated with 20 μl of each mAb at 4°C for 30 minutes. Following this incubation, the cells were washed in 2-3mls of ice cold PBS/azide to remove excess antibody and cells were collected by centrifugation at 1200 rpm for 3 min at 4°C. Stained cells were aldehyde-fixed by a 10 minute incubation in 0.5 ml of 0.2% paraformaldehyde at 4°C. Fixed cells were washed in 2-3 mls of PBS/azide, collected via centrifugation and resuspended in 0.5 ml of PBS/azide. Background fluorescence was determined using FITC, PE and PCP-conjugated goat anti-mouse antibodies as controls. Monocytes were excluded from the analysis by placing an electronic window around the lymphocyte population based upon their characteristic low angle forward and 90° light scatter properties.

Reagents

Recombinant vaccinia virus vectors. The vaccinia-EBV constructs used in these studies were provided by Dr. Elliot Kieff. A detailed description of the vaccinia virus constructs encoding the different EBNA genes (except EBNA-1) has been published previously (Murray et al., 1992) (Khanna et al., 1992) (Murray et al., 1990). All EBV sequences were derived from the B95.8 (type-1 or A) strain of the virus. Regarding the Vac-EBNA1 construct, the Ssp1 fragment
from the pTF7-5:EBNA1 plasmid was inserted into the pSC11 plasmid opposite the P7.5 promoter for EBNA1 expression. While EBNA1 expression is under control of T7 DNA polymerase, the baseline expression of EBNA1 is almost identical to that observed in B-LCLs (Khanna et al., 1991). All constructs contain sufficient genetic material to encode the relevant full-length EBV protein. A vaccinia construct containing no EBV latent genes and a dysfunctional thymidine kinase gene (Vac-TK-) was used as a negative control. Table 3 contains a complete list of vaccinia constructs used in these studies.

Established cell lines & corresponding MHC class-I haplotypes. See table 2.

Monoclonal antibodies & corresponding antigen specificities. See table 4.

Recombinant human lymphokines and growth factors.

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Use in Culture</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>rHu IL-2</td>
<td>In-vitro growth of cytotoxic T cells</td>
<td>Boehringer Mannheim W. Germany</td>
</tr>
<tr>
<td>rHu IL-4</td>
<td>Generation of B cell blasts</td>
<td>Genzyme, Cambridge, MA.</td>
</tr>
<tr>
<td>Acidic-Fibroblast Growth Factor</td>
<td>Establishment of primary human fibroblast lines</td>
<td>Sigma Immunochem. St. Louis, MO.</td>
</tr>
<tr>
<td>Basic-Fibroblast Growth Factor</td>
<td>Establishment of primary human fibroblast lines</td>
<td>U.S. Biochem. Co. Cleveland, OH.</td>
</tr>
</tbody>
</table>

**Immunobeads**: Rabbit anti-human IgM coated acrylamide beads. Anti-IgM coated acrylamide beads were used as a source of solid-phase stimulation for the generation of human B cell blasts. Beads (#15377H) were added to SRBC rosette negative B cell suspensions at a concentration of 2µg/ml and were purchased from Irvine Scientific, Santa Ana, CA.

**Miscellaneous Reagents**: AET-treated sheep red cells. Sheep red blood cells (SRBC) (Colorado Serum Co. Denver, CO.) were received in 60 ml aliquots which were diluted 1:2 in
RPMI-1640 and stored at 4°C for up to two weeks. The SRBC were treated with AET as follows: 120 mls of SRBCs were washed twice in several volumes of PBS and collected via centrifugation at 1200 rpm for 10 minutes. The washed SRBCs were resuspended in 125 mls of 37°C AET solution (see AET solution below) and incubated in a 37°C water bath for 15-20 minutes. If the AET/SRBC suspension did not darken, the pH of all solutions was checked and the procedure repeated. The red cells were then washed twice in PBS and pelleted at 1200 rpm (400 x g) for 5-10 minutes. The AET-treated red cells were then resuspended at a concentration of 10% vol/vol in RPMI-1640 containing 10% FBS (Sigma) and gentamicin (10μg/ml, Gibco).

2-aminoethylisothiouronium (AET) bromide solution was made as follows: Five grams of AET was dissolved in 100 mls of distilled water and the pH adjusted to 9.0 with 4N NaOH. The final volume was brought to 125 ml with distilled water and the solution sterile filtered through a 0.22 micron disposable filter system (Nalge Company, Rochester, NY.). This volume of AET solution was used to treat 20-40 ml of packed, washed SRBC or 120 mls of a SRBC suspension as received from Colorado Serum Co. The solution was used only on the same day it was made and excess AET solution was discarded as a several day old solution was observed to lyse the sheep red cells.

Rabbit complement. Lyophilized rabbit serum was used as a source of complement for monoclonal antibody/complement mediated depletion of specific lymphocyte subsets. The lyophilized rabbit serum was dissolved in 5-6 mls of plain RPMI-1640 and filter sterilized by passage through a 0.45μm filter before use. Reconstituted complement (#ACL3221) was freshly prepared for each set of depletions and was purchased from Accurate Chemical and Scientific Corp., Westbury, NY.

Skin biopsy kits. Human skin biopsies were obtained from healthy donors to generate primary human fibroblast cultures. Three and four mm skin biopsy punches were used to obtain biopsy material from the anesthetised lumbar region of HLA class-I typed donors. Biopsy punches (#443-50) were purchased from Baker Cummins Pharmaceuticals Inc., Miami, FL.
CHAPTER III
RESULTS

EBV strain typing by DNA-PCR

The infecting strain of EBV (type-1 or type-2) was determined for twenty-seven patients with a confirmed diagnosis of acute infectious mononucleosis. A representative assay is shown in Fig. 1: PCR amplified products from saliva and partially-purified B cells were hybridized with type-1 (panel a) or type-2 probes (panel b). PCR products from EBV positive cell lines, Namalwa (type-1), and Jijoye (type-2), were used as positive controls and the EBV negative cell line, Ramos was used as a negative control. No cross hybridization occurred and the patients were successfully typed. Of the 27 patients typed, 26 (>96%) carried EBV type-1 while only one patient (<4%), Dowda, was infected with type-2 EBV. A summary is presented in Table 1.

Isolation of T- and B-lymphocytes. AET-SRBC rosette selection was used to isolate highly enriched populations of T- and B-lymphocytes from all patient samples described in this study. SRBC-rosette selected T cells were used as a source of cytotoxic T cells and the non-rosette forming B-cell enriched fractions were used as a source of HLA-matched target cells. The SRBC-rosette positive population from AIM patients was analyzed by FACS and consisted mainly of CD3+ (95-98%), CD8+ (50-80%) T cells which were used as a source of effector cells in 51Cr release assays. The SRBC-rosette-negative population consisted mainly of CD19+ (80%) B cells which were used both to established EBV-transformed B lymphoblastoid cell lines (BLCL) and to generate B-cell blasts which were subsequently infected with recombinant vaccinia virus constructs and used as targets in the 51Cr release assays. Fig. 2 shows a representative set of FACS data for whole PBMC, rosette (+) and rosette (-) subpopulations.
Expression of single EBV gene products in target cells

**Immunofluorescence detection of EBV gene products.** Expression of specific EBV gene products in the various target cells was demonstrated by immunofluorescence. Typically, 85-95% of target cells infected with vaccinia constructs at an M.O.I. of 10 expressed high levels of the specific EBNA protein. Human fibroblasts infected for 12-16 hrs with vaccinia constructs encoding EBV nuclear antigens contained high levels of the specific EBNA protein as detected by immunofluorescence using pooled polyclonal human serum from EBV-seropositive /vaccinia-seronegative donors (Fig. 3c-3h). The EBV-negative T-cell line; CEM, served as a negative control and did not show the typical nuclear staining pattern characteristic of cells expressing one of the EBNA proteins (Fig. 3a). Fibroblasts infected with a control vaccinia construct Vac-TK-, containing no insert, also showed no nuclear staining (data not shown). EBV-transformed B-LCLs were included as positive controls which routinely displayed large, brightly stained nuclei along with thin, pale, unstained cytoplasm (Fig. 3b).

Human fibroblasts infected with recombinant vaccinia vectors encoding the EBV membrane protein LMP1, (Vac-LMP1), were stained with the LMP1-specific mAb S12. The kinetics of LMP-1 expression is shown in Figures 4c-4f. LMP1 was detectable as early as 4-5 hrs and levels rose steadily for over 12 hrs. Typically, protein levels rose for 24-36 hr following infection, but cell viability decreased rapidly at these later times (data not shown). An infection time of 12-16 hr was used to prepare target cells for $^{51}$Cr release assays as this length of infection allowed an optimum balance between levels of protein expression and cell viability. Fibroblasts infected for 12 hrs with the control vaccinia construct Vac-TK- served as a negative control (Fig. 4a). EBV-transformed B-LCLs were used as a positive control, which displayed pale, unstained nuclei along with the characteristic brightly staining patches on the cell membrane (Fig. 4b).

Expression of the major viral coat glycoprotein gp350 was demonstrated by staining
recombinant vaccinia vector infected fibroblasts with the gp350-specific mAb 72A1. See Fig. 4h for expression of Vac-gp350 and Fig. 4g for Vac-TK which served as a negative control. Several unsuccessful attempts were made to demonstrate expression of the Vac-LMP2A construct in target cells by immunofluorescence using pooled polyclonal human sera. A mouse mAb specific for LMP2A now exists but was not available for use at the time of these studies.

**Immunoblot detection of EBV gene products.** Expression of the proper sized EBV proteins encoded by the recombinant vaccinia vectors was demonstrated by Immunoblotting. Blots containing extracts from fibroblasts, MT2 lines and B-cell blasts infected with recombinant vaccinia constructs encoding EBNA proteins were probed with polyclonal human serum from EBV-seropositive/vaccinia-seronegative donors. Blots containing extracts from cells infected with vaccinia constructs encoding LMP1 were probed with the LMP1-specific mAb S12. Protein expression in the various cell types tested did not differ qualitatively nor quantitatively and thus only the results using B cell blasts are shown. Expression of EBNA1 in target cells was demonstrated as a single, faint band at the expected M.W. of 78 kDa, typically appearing at times greater than 16-20 hr following infection (Fig. 5a). Expression of EBNA2A was readily detected as a single, 86 kDa band at 8 hr post-infection and levels of protein increased over the 24 hr duration of the experiment (Fig. 5b). The vaccinia construct encoding EBNA-LP produced a series of bands of varying M.W. ranging from 20-45 kDa (Fig. 5c). Expression of the vaccinia constructs encoding EBNA3A, 3B, 3C and LMP1 all followed similar kinetics; a single band of the expected M.W. appeared at 4 hr post-infection and rose throughout the duration of the 24 hr experiment (Figs. 6a-6d).

Several unsuccessful attempts were made to demonstrate expression of the Vac-LMP2A construct by immunoblot using polyclonal human sera as the primary reagent. Under none of the various condition tested was there evidence that the protein was being properly expressed to any detectable level. Similarly, several attempts were made to demonstrate expression of the
Vac-gp350 construct using the gp350-specific mAb 72A1. On one occasion a band of approximately 200 kDa (unglycosylated gp350 = 220 kDa) was present in lanes containing extracts from Vac-gp350 infected cells which had not been heat denatured prior to electrophoretic separation (data not shown).

Cytofluorographic analysis of EBV-primed CTL

Primary EBV infection is accompanied by a profound expansion of peripheral blood lymphocytes, which gradually regress during convalescence. FACS analysis was utilized to more precisely describe specific changes in T cell subsets which accompany the maturation of cellular immunity to EBV.

Activation marker expression on CD8+ T cells during acute IM. The MHC class-II molecule HLA-DR is not expressed on resting T cells and thus, in the surface marker studies described here, was used as a marker to identify activated T cells. Similarly, CD11b (Mo-1) is a marker expressed on previously activated, but not naive, T cells and thus served here as a marker for long-term memory. Whole PBMC from acute IM patients and healthy controls were stained with fluorochrome conjugated mAbs specific for CD8, HLA-DR and CD11b and subjected to triple color FACS analysis. Selection of CD8+ cells was followed by two color separation of CD11b+ and HLA-DR+ cells. In comparison to healthy adult controls, acute IM patient PBMC contained a four to five fold increase in the percentage of activated CD8+/HLA-DR+ T cells (Table 5). Freshly isolated PBMC from acute IM patients also contain markedly elevated levels of CD8+/HLA-DR+/CD11b+ triple positive T cells which are not present in PBMC from healthy controls (Fig. 7). Triple color surface marker analysis performed on sequential PBMC samples (days 1, 4 and 8) from a representative acute IM patient (GRIBR) is shown in Fig. 8. The population of triple positive cells (CD8+/HLA-DR+/CD11b) reached peak levels very early, around day one (quadrant 2) followed by a relatively rapid and sustained loss of the HLA-DR
marker over time in all acute IM cases studied. Worthy of note is the sustained expression of CD11b resulting in a shift in the percent of double positive cells (CD8/CD11b) occupying quadrant 1 on day 8. The relatively rapid decrease in HLA-DR expression on CD8 T cells following the acute phase of primary EBV infection is easily observed when compared to a marker not expressed at high levels on the activated CD8 cells, such as CD57 (Fig. 9).

A summary of the mean percent of CD8 cells expressing HLA-DR and CD11b marker combinations on initial blood samples from seven acute IM patients and four healthy donors is shown in the table below.

<table>
<thead>
<tr>
<th>Surface Marker Combination</th>
<th>Acute IM Patients (n=7)</th>
<th>Healthy Controls (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total : CD8+/HLA-DR+</td>
<td>52.3% +/- 11.7</td>
<td>2.9% +/- 1.0</td>
</tr>
<tr>
<td>CD8+: HLA-DR+/CD11b+</td>
<td>46.4% +/- 8.9</td>
<td>2.3% +/- 2.1</td>
</tr>
<tr>
<td>CD8+: HLA-DR-/CD11b+</td>
<td>13.1% +/- 6.7</td>
<td>8.2% +/- 6.1</td>
</tr>
<tr>
<td>CD8+: HLA-DR+/CD11b-</td>
<td>22.4% +/- 8.7</td>
<td>9.9% +/- 3.1</td>
</tr>
<tr>
<td>CD8+: HLA-DR-/CD11b-</td>
<td>18.0% +/- 8.1</td>
<td>79.7% +/- 6.1</td>
</tr>
</tbody>
</table>

Quantitative changes in the percentage of cells expressing each surface marker were also followed over time. The mean percent change in activation marker expression on CD8 cells from three acute IM patients, over a 10-12 day time period, is shown in figure 10. While the percentage of CD8 cells expressing HLA-DR dropped by greater than 30% over 10-12 days, the percentage of CD8 cells expressing CD11b (Mo1) dropped by only 5%. The net result is a decrease in the size of the activated, cytotoxic T-cell pool (CD8+/DR+/CD11b+) and an increase in the size of the long-lived, resting, memory T-cell pool (CD8+/DR-/CD11b+).

These findings were extended by independent two color FACS analysis of HLA-DR and CD11b expression on CD8+ T cells from three groups of individuals including: 1) acute IM patients at the earliest stage of EBV infection, 2) acute IM patients at 6 weeks following initial
presentation with symptoms, and 3) EBV seropositive individuals at greater than 6 months post primary EBV infection. The results shown in Fig. 11, demonstrate a significant drop in HLA-DR expression at 6 weeks following acute IM, whereas a similar drop in CD11b (Mo-1) expression did not occur until some time between six weeks and six months. This data appears to support the theory that CD11b represents a marker for long-lived immune memory cells, and will be described more fully in the discussion section.

**Activation marker expression on in-vitro primed memory CTL.** The percentage of in-vitro restimulated CTL which express HLA-DR (60-70% positive) was found to be similar to that of the in-vivo primed CTL, however, unlike the in-vivo activated CTL, expression of Cd11b was essentially absent from the in-vitro primed CTL. In fact, levels of CD11b on in-vitro propagated CTL were observed to drop by 80% in as little as 8 days in culture (data not shown). Thus CD11b is not an accurate marker for identifying memory T cells propagated in vitro.

**EBV-specific HLA-restricted CTL in peripheral blood of EBV-seropositive donors**

When freshly isolated, whole PBMCs from EBV-seropositive donors were used as effector cells in $^{51}$Cr-release assays, target cell lysis occurred independent of the presence of EBV antigens or HLA class-I molecules on target cells. In Fig. 12a, the NK-sensitive targets K562-neo (HLA$^-$) and K562-A2 (HLA-A2) were lysed in direct proportion to the number of SULJO PBMC (effector cells) added. In contrast, the EBV-infected cell lines SULJO-BLCL (autologous) and BYRKE-BLCL (allogeneic) were not lysed to any measurable extent even at the highest E:T ratio of 50:1.

In contrast, when whole PBMC from EBV-seropositive donors were restimulated in-vitro with the autologous BLCL, lysis of HLA-matched EBV-infected cells increased relative to the non-specific lysis of EBV-uninfected, HLA-mismatched targets. A representative
experiment is shown in Fig. 12b. In addition to killing the NK-sensitive targets K562 and HSB-2, the BLCL-restimulated PBMC (bulk CTL culture) also lysed the EBV-infected, HLA-matched autologous BLCL in preference to the EBV-negative (MT2) or HLA-mismatched targets (Allo). The specificity of target cell lysis by these bulk CTL cultures was further improved by enriching the culture for T cells, and depleting NK cells prior to in-vitro restimulation. The results in Fig. 12c show that target cell lysis was restricted to the autologous BLCL (EBV infected, HLA class-I matched) and that all HLA-mismatched targets (Allo, K562, HSB-2), regardless of EBV status, were not lysed. Attempts to generate EBV-specific CTL lines from two available EBV-seronegative donors resulted in the expansion of a population of effector cells which mediated low levels of non-specific target cell lysis in an EBV antigen independent and non-HLA class I restricted manner. See Fig. 12d. Thus, EBV-seropositive individuals have EBV-specific CTL in their peripheral blood which can be grown in-vitro by stimulation with autologous BLCLs.

**EBV-specific CTL lyse fibroblasts and B cell blasts expressing single EBV genes.** EBV-specific CTL cultures were established from EBV-seropositive donors and tested as effector cells in $^{51}$Cr release assays against various target cell types infected with recombinant Vac-EBV constructs. Fibroblasts and B cell blasts infected with vaccinia constructs encoding the EBV latent proteins (EBNA-3A, 3B, 3C, LMP-1) were specifically recognized by the EBV specific memory CTL, Figs. 13a-d. Additionally, autologous BLCLs (positive controls) were efficiently lysed, while allo-BLCLs, uninfected target cells, and targets infected with the vaccinia construct Vac-TK (negative controls) were not significantly lysed. Both the fibroblasts and B cell blasts expressed the introduced EBV genes and were lysed by EBV-specific CTL, although, the B cell blasts proved to be the easiest target cells to work with, as they are quickly and easily generated in large numbers.
In-vivo activated CTL derived directly from the blood of acute IM patients display EBV-specificity and HLA-restriction in-vitro

**Target lysis by acute IM CTL is antigen-specific and HLA class-I restricted.** Whole PBMC from acute IM patients contain large numbers of in-vivo activated CD8+ CTLs which do not require in-vitro restimulation prior to their use as effector cells. Bulk T-cell enriched preparations containing primarily activated CTLs were obtained by SRBC-rosette selection from whole PBMCs. Non-HLA-typed effector cell populations prepared in this manner mediated high levels of killing against EBV-infected BLCLs in preference to EBV-uninfected, HLA-identical T-cell lines (MT2) Fig 14a. These crudely prepared, bulk CTL cultures still contain significant numbers of NK cells and thus also mediate high levels of NK-like killing against the NK-sensitive target K562, Fig 14a.

In-vivo activated, SRBC-rosette selected CTL preparations derived from HLA-typed acute IM patients mediate higher levels of lysis against HLA-matched than HLA-unmatched BLCL targets, Fig 14b. Removal of NK cells from the T-cell enriched population in Fig 14b, provided a CTL preparation which mediated lysis of only EBV-infected, HLA-matched target cells (Aliar-BLCL) Fig 14c, and not EBV-infected, HLA-mis-matched (BLCLs) or HLA-negative targets (K562). This target cell lysis is directed against EBV antigens and not allo-derived structures, as this same bulk CTL preparation specifically lysed only Aliar-derived B-cell blasts (Allo) infected with Vac-EBV constructs expressing EBNA-3B and LMP-1 Fig 14d.

These activated CTLs are highly IL-2 dependent and can be maintained in culture for several weeks in the presence of rIL-2 (20U/ml) alone. Non-specific stimulation by anti-CD3 in addition to rIL-2 allowed long term (several months) maintenance of these activated T cells.

**CTL from Acute IM patients lyse target cells expressing single EBV genes.** The specificity of the primary CTL response to EBV infection was evaluated in 35 acute IM patients.
Enriched populations of in-vivo activated, CD8+ CTL from all 35 patients recognized and lysed autologous B cell blasts expressing single EBV gene products. In-vivo primed CTL recognized all eight EBV latent proteins, including EBNA-1 and the lytic cycle glycoprotein gp350. Primary CTL responses were frequently directed against as many as 4-5 EBV proteins, with greater than 90% of responses including one or more of the EBNA-3 proteins, and 60% recognizing EBNA-3C alone. Importantly, nine of the 35 patients examined had CTL which lysed Vac-EBNA1 infected targets, and similarly, 7 of the 35 patients had CTL which lysed Vac-gp350 infected targets. A composite representing the spectrum of primary CTL responses to EBV from six individual acute IM patients is shown in Fig. 15, and details of CTL responses for all 35 patients is included in table 6. Percent specific lysis values for patients with significant cellular immune responses against EBNA-1 are included in table 7, and similarly, results for gp350 specific lysis are in table 8. Minimum criteria for significant target cell lysis included; spontaneous 51Cr release from targets < 25%, and individual target cell lysis had to exceed Vac-TK (negative control) lysis by > 10% in order to be considered significant.

In-vivo primed, EBV-specific CTL are CD8+/class-I restricted. The cellular phenotype of the in-vivo primed effector cells used in the above 51Cr release assays were shown by monoclonal antibody (mAb) blocking experiments to be CD8+/Class-I-restricted. Anti-class-I antibody (BBM.1) treatment of target cells reduced lysis by an average of 50-80% (Fig. 16a) while mAb directed against class-II molecules on target cells had no such effect (data not shown). Similarly, anti-CD8 mAb treatment reduced target cell lysis by an average of 40-70% (Fig. 16b) while mAb against CD4 molecules had little or no effect (data not shown).

Determination of cell surface phenotype of the in-vivo primed effector cells by FACS analysis confirmed the mAb blocking studies described above. Representative data from acute IM patient "Marro" are shown in Fig. 17. As depicted here, all T cell enriched cultures used as
effector cells contained a major T cell subset (60-80%) which stained with mAb to CD3/CD8. A more complete phenotype analysis of effector cells, including activation markers, is included in section F of the results.

In-vitro primed memory CTL from convalescent IM patients recognize predominantly EBNA3 antigens. The memory/secondary CTL response to EBV was evaluated in 32 acute IM patients at 6-8 weeks following primary infection. PBMC from convalescent donors do not contain significant numbers of activated, EBV-specific CTL, and thus all PBMC cultures were restimulated with the autologous BLCL prior to use as effector cells. Target cells consisted of autologous B cell blasts infected with vaccinia constructs encoding single EBV genes. In contrast to the primary response, memory CTL responses measured in individual patients were generally restricted to one or two EBV antigens, and usually included one of the EBNA3 proteins. A composite of representative results from four patients is shown in Fig. 18. At the two extremes, some patients appeared to have identical primary and secondary CTL responses to EBV (Figs. 19a-b) while others demonstrated markedly different primary and secondary CTL responses (Figs. 19c-d).

In contrast to the primary T cell response, no memory CTL responses were detected against gp350 and only one of 32 patients had memory CTL which displayed significant lytic activity against EBNA-1 expressing targets. The primary CTL response by this patient was directed against EBNA 3A, 3C and LP, while the memory response was directed against EBNA 1, 3A, 3C and LP (Figs. 20a-b). These unique results, while potentially very important, could not be repeated as no further PBMC samples were available from this patient. A complete list of acute IM patient primary and secondary CTL responses is shown in Table 6, together with a graph comparing the percentage of patients responding to a given EBV antigen in primary vs secondary CTL responses (Fig. 21). These results demonstrate that the primary and secondary CTL responses to EBV are marked by the overwhelming immunodominance of proteins in the
EBNA3 family (EBNAs 3A, 3B, and 3C).

Recombinant vaccinia-EBNA1 stimulated CTL

Vac-EBNA1 stimulated CTL from EBV seropositive/vaccinia seropositive donors. In order to preferentially stimulate and expand EBNA1-specific memory CTL, PBMC from four EBV-seropositive/vaccinia seropositive donors were primed with autologous B blasts or BLCLs infected with a recombinant vaccinia vector encoding EBNA-1. CTL responses when stimulated with either Vac-EBNA1 infected B blasts or Vac-EBNA1 infected BLCLs were directed against either vaccinia gene products or to EBV in general, with little or no clear indication of an EBNA1-specific CTL response. Representative data shown in Fig. 22a were generated using CTL stimulated Vac-EBNA1 infected B blasts and demonstrate lysis of all target cells infected with either control (TK) or EBNA-1 encoding vaccinia constructs (E1). The two autologous targets not infected with vaccinia constructs were not lysed to any significant degree, indicating a primarily vaccinia dominated CTL response. The results shown in Fig. 22b were generated with CTL which had been stimulated with autologous BLCLs infected with the Vac-EBNA1 construct. In this case the CTL lyse both the vaccinia infected as well as vaccinia uninfected BLCLs. In contrast, only the vaccinia infected B blasts and not the uninfected B blasts were lysed. These results indicate that the CTL response was directed against both vaccinia and EBV encoded antigens, again, with no clear preference for EBNA1 expressing targets.

Vac-EBNA-1 stimulated CTL from acute IM patients. Restimulation of PBMC from acute IM patients was accomplished in a similar manner, with the exception of the timing of exogenous IL-2 addition. Activated CTL from acute IM patients are IL-2 dependent and were thus supplemented with IL-2 at the initiation of cultures, unlike PBMC from normal donors which were deprived of IL-2 for the first 5-6 days of in-vitro stimulation. Freshly isolated PBMC from acute IM patients were stimulated with Vac-EBNA1 infected B blasts and target
cell lysis was evaluated with a variety of vaccinia infected and uninfected targets. Results from a representative experiment using PBMC from a vaccinia-seronegative, acute IM patient Levro are shown in Fig. 23. Lysis of the autologous B blasts and BLCLs increased by 8.6% and 12.5% respectively, when infected with the vaccinia construct encoding EBNA 1, as compared to the control vaccinia construct Vac-TK. It is worth noting that freshly isolated PBMC from this acute IM patient did not initially demonstrate significant lysis of EBNA1 expressing targets over the control Vac-TK infected targets. The memory CTL response to EBV measured in this patient was directed to EBNA3C and was included here for the purpose of comparison.

**Identification of antigen source by cold target competition assay.** In an effort to further expand the apparent EBNA1-specific component of the "Levro" bulk CTL culture (shown in fig. 23), three additional stimulations were carried out with Vac-EBNA1 infected B blasts. The target cell specificity of this culture was evaluated in a cold target competition assay. Radiolabeled "hot" targets consisted of autologous B blasts infected with either the Vac-TK (control) or Vac-EBNA1 constructs. Cold competitor cells consisted of unlabelled, autologous B blasts infected with the Vac-TK construct. Cold competitor cells were mixed with 51Cr labeled targets at a ratio of 10:1 and the Vac-EBNA1 expanded, effector CTL were added at ratios of 1:1 or 5:1. The Vac-EBNA1 expanded CTL lysed both the Vac-TK and Vac-EBNA1 targets equally well and likewise the cold target competitors reduced lysis of both targets by 50-70% (Fig. 24a). In order to determine whether EBV expressing targets could serve as cold competitors, a similar assay was performed using a panel of partially HLA-matched BLCLs as cold competitors. The results shown in Fig. 24b demonstrate that as above, the majority of target cell lysis was directed to vaccinia encoded antigens. Vac-TK infected cells served as efficient cold competitors whereas the EBV infected BLCLs did not prevent lysis of the chromium labeled targets.

The results of standard chromium release assays and cold target competition assays
demonstrate that target cell recognition by Vac-EBNA1 expanded CTL was dependent upon vaccinia encoded antigens. Classically, virus specific T cells provide long term immunity to viruses but, this vaccinia specific, in-vitro stimulated response was obtained with PBMC from a vaccinia seronegative donor, leaving to question the identity of the cells mediating target cell lysis. Dual color FACS analysis revealed that the Vac-EBNA1 expanded effector cell culture consisted primarily of CD3+/CD4-/CD8-/CD16-/CD19-/HLADR+/CD45RO+/TCRγδ+/TCRδγ+ T cells. The table below summarizes the percentages of cells bearing selected CD markers and the FACS contour plots are contained in Figs. 25-26.

<table>
<thead>
<tr>
<th>CD Markers</th>
<th>% Cells Stained</th>
<th>CD Markers</th>
<th>% Cells Stained</th>
</tr>
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<tr>
<td>CD3+</td>
<td>95.7</td>
<td>CD3+/TCRαβ+</td>
<td>0.9</td>
</tr>
<tr>
<td>CD19+</td>
<td>0.1</td>
<td>CD3+/TCRαδ-</td>
<td>96.0</td>
</tr>
<tr>
<td>CD16+</td>
<td>0.8</td>
<td>CD3+/TCRγδ+</td>
<td>83.8</td>
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<td>27.0</td>
<td>CD4+/TCRγδ+</td>
<td>0.8</td>
</tr>
<tr>
<td>CD3+/CD4+</td>
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<td>CD8+/TCRγδ+</td>
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<tr>
<td>CD3+/CD8-/CD4+</td>
<td>66.5</td>
<td>CD16+/TCRγδ+</td>
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</tr>
<tr>
<td>CD3+/CD56+</td>
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<td>CD8+/CD56+</td>
<td>17.5</td>
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<td>89.6</td>
<td>CD8-/HLADR+</td>
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CHAPTER IV
DISCUSSION

Importance of CTL immunity against EBV. Experiments of nature have demonstrated that individuals lacking the ability to produce antibody handle most viral infections quite well, but are significantly more susceptible to bacterial infections. In contrast, individuals with genetic deficiencies in their ability to mediate cellular immune functions are unusually susceptible to a variety of viral infections. The latter condition directly pertains to EBV as exemplified by nature's lessons with "David the bubble boy" (XSCID) (Rennie, 1993) and the now well described Duncan family of XLP males (Purtiø et al., 1975) (Sullivan and Woda, 1989).

While CTL play a major role in the control of acute viral infections, most immunization strategies aim to induce neutralizing antibody against glycoprotein or structural antigens present on the virus coat. The effectiveness of this approach is limited in the setting of persistent/latent infection, where virus is cell associated and the major viral coat glycoproteins are generally not expressed. Thus, vaccine strategies to prevent persistent infection should aim to induce CTL-mediated immunity against viral-encoded latent proteins. In this regard, a decade has now passed since the initial description of antigen recognition by CTL, involving proteolytic fragments of viral proteins associated with cell surface HLA class-I molecules (Townsend et al., 1986), and since that time, virus encoded, 9-12 amino acid peptide-epitopes have been described for many viruses (Oldstone, 1991), including EBV (Masucci et al., 1993).

Accordingly, CTL are well suited to control persistent infection, as they posses the capacity to mediate lysis of virally infected cells with very low levels of cell surface-associated viral antigen. In fact, CTL likely require no more than 100-500 viral peptide-MHC class-I complexes for activation and lysis (Demotz et al., 1990) (Christnick et al., 1991). Importantly, CTL are effective against many immediate-early or early viral proteins which
are transcribed prior to structural proteins and virus assembly (Oldstone, 1989). Thus, the ability of CTL to destroy latently infected cells is particularly useful in the elimination of these potential virus factories.

Since the increase in number of circulating CD8+ lymphocytes during acute IM correlates closely with a rapid decline of EBV infected B cells in the peripheral blood, it was of interest to identify the EBV encoded antigens recognized by cytotoxic T-cells which control the spread of Epstein-Barr virus during primary infection. To this effect, we studied the development of virus-specific CD8+ lymphocyte responses in college students with acute infectious mononucleosis. Following a prodromal period of several weeks, newly EBV infected patients present with signs and symptoms of acute IM and their peripheral blood contains greatly elevated levels of activated CD8+ T cells which, like the memory CTL response to EBV, we now know is HLA class-I restricted and directed at EBV latent gene products. Our results represent the first such description of the EBV-specific CTL responses in the setting of acute infection, which will be useful in the rational design of an EBV vaccine.

**Primary vs memory CTL responses.** The medical interest in EBV is based upon the ability of the virus to cause both severe acute infections as well as lymphoproliferative diseases. The aim of the research presented here is to determine the origin of antigenic peptides for the induction of EBV immunity by utilizing vectors encoding individual EBV latent genes. Thus, in searching for viral epitopes with relevance for the immune control of acute and chronic EBV infection, we first gave consideration to the stage of viral replication during which the antigens which elicit protective CD8 T lymphocyte responses are likely to be expressed. The criteria for defining immunodominant antigens of a large virus such as EBV require the application of several fundamental principles, including (1) the generation of CTL by natural *in vivo* infection followed by antigen-free propagation of sensitized CD8 T lymphocytes with IL-2; and (2) batch testing of a defined panel of viral proteins for their
capacity to sensitize target cells to CTL-mediated lysis. Unfortunately, the importance of the first principle is frequently ignored, which may lead to a false impression of the actual immunodominant T cell response to a pathogen.

Our results demonstrate that during primary EBV infection, the CTL-mediated response is directed against all 8 latent proteins, including EBNA-1 (7 of 35 patients). To our knowledge, these results represent the first systematic analysis of EBV-specific CTL during acute IM. Importantly, with regard to vaccine development, greater than 90% of acute IM patient CTL recognize one or more EBNA3 proteins, and a full 60% recognize EBNA-3C.

Our data also show that 20% of acute IM patients have CTL which lyse gp350 expressing targets. This finding is not all that surprising given the high levels of virus replication, and thus gp350 expression, during primary EBV infection. Additionally, several previous studies have verified the role of T cells in inhibiting cells expressing EBV replicative antigens, including gp350 (Bejarano et al., 1988) (Bejarano et al., 1990). The most likely explanation for the paucity of reported gp350-specific CTL activity involves the methodology used to generate activated CTL effector cells. Virtually all EBV-encoded T cell epitopes defined to date have been identified using in-vitro re-stimulated memory CTL rather than strictly in-vivo activated EBV-specific CTL. Importantly, memory CTL lines generated by in-vitro stimulation with the autologous BLCL are not exposed to gp350, as the major virus coat glycoprotein gp350 is not expressed in latently infected B cells.

Our results describing the memory CTL response to EBV are consistent with those reported by other groups (Masucci et al., 1993), and show that a given individual's CTL response to EBV is typically a composite of reactivities directed against a variety of latent antigens. In contrast to the primary cellular immune response to EBV, we found the memory CTL response to be directed against a more restricted group of EBV antigens. While most CTL responses were directed against the higher molecular weight nuclear antigens, EBNA3A, 3B, and 3C, CTL also recognized EBNA2, and LP in addition to LMP-1 and -2, albeit at lower frequency than
the EBNA-3 family. Thus, while the EBNA-3 proteins were clearly immunodominant here also, an EBNA-1-specific memory response was observed in only one patient, and no gp350-specific CTL responses were detected, likely a result of BLCL-mediated stimulation (BLCL do not express gp350).

Memory T cell responses to EBV were evaluated in 32 patients, only one of which demonstrated measurable cytotoxic activity against EBNA-1 expressing targets. Curiously, this patient did not demonstrate an EBNA-1-specific primary CTL response. Unfortunately, this patient dropped out of the study for unknown reasons and PBMC were not available to confirm these results. In a somewhat analogous situation, while many patients had very similar primary and memory CTL responses, a significant number of individuals demonstrated only minimally overlapping primary and memory CTL responses. These observed differences may reflect a combination of in-vivo "affinity maturation", or more correctly, avidity selection, toward the immunodominant EBNA-3 family, which may be further promoted by re-stimulation in-vitro with the autologous BLCL.

In general, the overall levels of lysis were typically greater in primary CTL assays as compared to memory CTL assays, while net lysis, or "specific lysis" was usually greater in the memory CTL assays. We attribute this difference to the higher levels of background (non-specific) lysis present in the primary CTL assays. Importantly, the same criteria for determination of significant lysis were applied to both primary and memory CTL assays; ie. (1) spontaneous $^{51}$Cr release <25% (usually <10% for most assays), and (2) specific lysis >10% above background (Vac-TK) lysis.

As stated earlier, our findings are in keeping with the concept that the T cell response to a given protein or pathogen is frequently focused on one to three epitopes (Adorini et al., 1988) (Perkins et al., 1991), however, it is not known whether this immunodominance is a reflection of unequal presentation of peptide/MHC complexes by infected cells, or strictly a matter of TCR affinity for various Ag/MHC combinations. The discrepancy between our
measured primary and memory CTL responses to EBV may be a reflection of the finding that changes in peptide antigen/MHC class-I density during the course of viral infections can serve to modulate T cell responses. While, relatively strong CTL responses may occur during periods of extensive expression and presentation of viral antigen, during the later phases of immune responses, CTL activity to a given antigen may be reduced as a consequence of lower concentration. This concept also applies to the role of CD8 in CTL recognition, which shows an inverse correlation with Ag/MHC density requirements (Shimonkevitz et al., 1985). Here, the selection of in-vivo protective memory CTL bearing very high avidity TCR can occur independent of CD8, which allows for later recognition of virus infected cells expressing low levels of Ag/MHC complexes.

In addition to the quantitative effects of Ag/MHC concentration on CTL activation just described, qualitative changes in Ag/MHC complexes have also been suggested to influence CTL activity (Matko et al., 1994). While clustered HLA class-I molecules are readily detected on activated normal B and T cells, as well as B and T lymphoblasts lines, class-I clusters are absent from resting B and T cells. This has been interpreted to suggest that activation induced HLA-clustering may partially explain why both anti-viral and allo-specific CTL are able to kill lymphoblasts and virus transformed cells more readily than resting lymphocytes. In this context, it would be interesting to determine whether latency type-III EBV-transformed B cells have cell surface HLA-clusters while latency type-I EBV-transformed B cells in vivo, have only HLA-monomers, contributing to immune evasion.

A number of more specific immune mechanisms exist which may account for our measured discrepancy between primary and memory CTL responses to EBV. For example, peripheral deletion of EBNA1-specific CTL may occur during the exuberant primary CTL response to EBV, which is plausible given that activated CD45RO+ T lymphocytes in acute infectious mononucleosis lack bcl-2 expression and undergo apoptotic cell death (activation-
driven deletion) (Uehara et al., 1992) (Tamaru et al., 1993). In fact, during acute EBV infection, the predominant subset of cells in the peripheral circulation are CD45RO+/CD8+/bcl-2-. Importantly, many of these apoptosis prone, activated CTL can be rescued from cell death by addition of exogenous IL-2 to the in vitro culture medium, resulting in increased expression of bcl-2 (Akbar et al., 1993). If EBNA-1-specific CTL do exist transiently during the primary immune response, exactly why they would be preferentially deleted later on remains unknown, but may be a result of the persistent and almost exclusive expression of EBNA-1 antigen by latency type-I EBV-transformed B cells (covered in detail later).

In contrast to peripheral deletion, the CTL may persist in an anergic state, as suggested by one group who has shown that with respect to anergy, many mature CD8+ T cells initially proliferate extensively, and then die or become anergic and remain refractory to TCR stimulation, even in the presence of exogenous IL-2 (Rocha et al., 1993). Additionally, it has been demonstrated that anergy is maintained in vivo unless the inducing antigen is removed (Ramsdell and Fowlkes, 1992), and that clonal anergy of mature T cells can be reversed in the absence of antigen (Rocha et al., 1993). A convincing example of this phenomenon involves an experiment in which peripheral T cells from female anti-HY TCR transgenic mice are transferred to syngeneic nu/nu male mice (Rocha and von Boehmer, 1991). The recipient mice have no T cells of their own, allowing the behavior of the transferred T cells to be readily studied. The HY-specific T cells, which recognize HY in the recipient males, initially proliferate for five days followed by the disappearance of most but not all cells. The remaining HY-specific T cells are nonresponsive to TCR-mediated signals, even in the presence of added IL-2. Thus, these cells become essentially unresponsive or anergic following an initial antigen induced growth phase. Importantly, when the remaining anergic cells are transferred back to female mice for prolonged periods of time (i.e. antigen free mice), HY responsiveness returns, implying that persistent stimulation with antigen is required to maintain this nonresponsive state. Obviously, removal of antigen is an unlikely event for cells anergic to an
authentic self-antigen. Thus it is somewhat perplexing that B7 transfection of self-tumors also induces immunity to self-tumors lacking B7 (Chen et al., 1992) (Townsend and Allison, 1993). Could this represent reversal of the anergic state? In this context, the persistent expression of EBNA 1 in small, resting B cells following primary infection, may both induce and maintain T cell anergy to this viral protein. The absence of B7 on latency type-I EBV transformed B cells in vivo makes the concept of EBNA-1 anergy induction all the more appealing.

In the setting of acute viral infection, the distinction between anergy and peripheral deletion may not be necessary, as clonal anergy has been suggested to represent a prelude of the continuum leading to clonal elimination (Kroemer and Martinez, 1992) (Schwartz, 1992). Functionally, most forms of tolerance probably represent clonal deletion, with or without a preceding proliferative phase, and in this sense, clonal deletion rather than anergy, may better explain the disappearance of EBNA1-specific CTL activity following the primary response, especially in light of evidence suggesting that some energized CD8+ clones retain their cytolytic ability (Otten and Germain, 1991) (Go et al., 1993). Thus, during TCR recognition of antigen, positive and negative signals must be balanced in order to guide cell survival or cell death, as immunologic memory against other EBV proteins is retained following acute infection. At present, the mechanism which allows in vivo activated T cells to return to a resting state and survive is unknown. One possible explanation has been offered involving signaling through the B7/CD28 costimulatory pathway, which is analogous to the survival of germinal center B cells following surface Ig crosslinking by CD40 (Liu et al., 1991).

Thus, how are the EBV-specific memory cells selected? One step toward answering this question comes from evidence mentioned earlier indicating that the relative sensitivity of CD8+ T cells to inhibition with anti-CD8 mAb provides a rough measurement of TCR affinity for Ag/MHC (MacDonald et al., 1982). In general, most CTL recovered during the height of the primary immune response are readily inhibited by mAb to CD8, suggesting a predominantly low affinity CTL response. This concept is also in keeping with our results. In contrast to the
primary response, the majority of CD8 T cells recovered several months following the initial antigen priming are relatively resistant to inhibition by anti-CD8 mAb. Accordingly, unlike many of the CTL present during the primary immune response to a virus, memory CTL appear to have higher avidity interaction with target Ag/MHC structures, which function more or less independent of CD8 (Hill et al., 1992). However, in contrast to B cells, T cells do not undergo affinity maturation by somatic hypermutation, but rather appear to mature by preferential survival of CTL bearing high affinity TCRs. Application of this concept to primary EBV infection would suggest that the EBNA-1-specific CTL present during primary infection merely represent short-lived, low-affinity CTL.

The differences in TCR affinity of primary vs memory CTL described above, have a direct bearing upon the interpretation of data generated using these effector cell populations. Accordingly, one should consider whether it is correct to refer to lymphocytes prepared at the end of the primary response to infectious virus as newly formed memory cells, or are they more properly termed effector cells? In a review by J. Sprent, the author suggests the latter term of effector cells is more accurate (Sprent, 1994). Likewise, lymphocytes recovered several weeks after the primary immune response should be referred to as a mixture of effector and memory cells. Here, one is confronted with the dilemma of phenotypically distinguishing between these two cell types. The difficulty arises because memory T cells display many of the surface activation markers present on effector T cells. A rather popular theory invoked to explain this phenotypic similarity would suggest that memory T cells actually represent semi-activated cells engaged in low-grade responses to persisting antigen.

Currently, the debate over the importance of antigen in maintaining long-term memory remains unresolved. Direct evidence that memory depends on antigen persistence comes from the reported rapid decay (days to weeks) in memory responses when primed lymphocytes are transferred to antigen naive hosts (Gray and Matzinger, 1991) (Oehen et al., 1992). Importantly, antigen introduction into the naive host results in the maintenance of memory.
Still others, have suggested that the paradox of why CD45RO+ population contains the primed/memory CTL pool yet expresses low levels of bcl-2 and are susceptible to apoptosis, can be reconciled by the theory that continuous restimulation of memory T cells is necessary to increase bcl-2 expression (Akbar et al., 1993). Equally convincing evidence that long-term survival of CD8+ memory cells can occur in the absence of antigen comes from unpublished data by Jamieson and Ahmed (Sprent, 1994). The authors have demonstrated that LCMV-specific CTL taken 3 months after antigen priming survive greater than 1 year following adoptive transfer to LCMV naive mice, and show little or no decrease in precursor frequency. Given that the majority of evidence suggesting maintenance of memory requires continued presence of priming antigen rests largely on studies with cells taken within 2 months of priming, it may be more accurate to suggest that antigen persistence appears necessary for the survival of effector/early memory cells but may not be required for long-term memory cell survival.

Similarly, Rocha et al. have demonstrated that in the absence of antigen, anergic T cells can persist for several months in recipient mice, which subsequently convert to functional activity and behave like memory T cells (Rocha et al., 1993).

The memory response to EBV includes CTL specific for all of the EBV genes expressed in latently infected cells, with the exception of EBNA1. The generally accepted view to explain this finding proposes that latency type-I EBV-transformed B cells in vivo serve as a immunologically protected reservoir for EBV persistence and reactivation. What remains unknown is why these cells are nonimmunogenic. Consequently, cells which escape the tightly restricted EBV expression characteristic of type-I EBV latency, proceed to type-III latency and express a broader array of EBV latent protens which serve to restimulate and thereby maintain the memory population of EBV-specific CTL. Based upon the data contained in this dissertation, one might extend this hypothesis to include the concept that type-I latently infected B cells, which lack cell surface B7, serve as a constant source of costimulator negative EBNA1 antigen which maintain an EBNA1 nonresponsive state.
In order to explain EBV persistence, numerous investigators have speculated for over a decade on the absence of T cell epitopes encoded by EBNA1. The data in this dissertation along with the recent description of an EBNA1-specific CD4+ CTL clone (Khanna et al., 1994) suggest otherwise. We cannot state with certainty the fate of EBNA1-specific CTL present during the primary immune response to EBV, but this work does demonstrate the importance of interpreting findings strictly within the context under which they are derived. In other words, experiments performed with in-vitro primed CTL have discounted a role for EBNA-1-specific CTL which may not accurately reflect in-vivo immune events. This message is nicely captured in a review titled 'subversion of the immune system by pathogens' (Marrack and Kappler, 1994), "we learned a lot about the properties of immunity to materials such as sheep red blood cells, ...most of the principles that are the foundation of modern immunology were learned with these models...however, ...in real life, most infectious organisms have spent their millions of years of coevolution with the immune system developing mechanisms of manipulating the system. The upshot is that no invading organism behaves exactly like a sheep red blood cell and, if immunologists really want to understand how infectious diseases interact with their hosts, they have to study the disease and host themselves. Artificial substitutes simply will not do." In fact, the over-expression of EBV genes via vaccinia vectors fits right into this category.

An appropriate example reflecting the accuracy of in-vitro models is the recent demonstration of discrepancy between in vitro measurable and in-vivo virus neutralizing CTL activity (Speiser et al., 1992). The authors set out to determine the in-vivo relevance of T cells exhibiting crossreactivity in-vitro and the respective role of the TCR affinities involved. Analysis of high and low avidity T cell activities both in-vivo and in-vitro, demonstrated highly specific class-I restricted CTL responses in-vivo and lysis of virus infected target cells in-vitro. In contrast, these same CTL were MHC crossreactive and less discriminatory against targets expressing high MHC/Ag densities (peptide pulsed) in proliferative assays. Thus,
relatively high affinity TCR are required for virus neutralization in-vivo, while in-vitro T cell responses to peptide coated targets involve engagement of low avidity TCR with broader specificity, calling into question their biological relevance. One possible explanation for this phenomenon involves the unphysiologically high concentration of peptides presented during in-vitro assays (Milligan et al., 1990).

Importantly, the above results also demonstrate a correlation between TCR avidity and the density of antigen/MHC class-I complexes. This is not surprising, given that the avidity of T cells for their targets is a direct result of TCR and accessory/adhesion molecule interactions. In fact, in-vitro measured CTL activities must be interpreted cautiously, as low avidity TCR/MHC-Ag specificities may be detected under certain experimental in vitro conditions which are below the physiologically relevant threshold required for in-vivo CTL function. Accordingly, while it is possible that low avidity, crossreactive T cells provide some antiviral protection in vivo, it is equally plausible that they merely represent biologically irrelevant, in-vitro measurable T cell activities. Thus, knowing that MHC/Ag density can influence T cell responses, we must at least consider this as a possible explanation for the EBNA1-specific CTL activity measured in our in-vitro primary CTL assays. As stated earlier, CTL likely require no more than 100-500 viral peptide-MHC class-I complexes for activation and lysis (Demotz et al., 1990) (Christnick et al., 1991) and importantly, type-I latently-EBV-infected B cells in vivo express very few EBNA-1 molecules (10-20).

Low levels of EBNA-1 protein in-vivo may also promote immune escape as the degree of CTL degranulation has been demonstrated to be directly proportional to peptide/MHC density (Kane and Mescher, 1993). It appears that empty class-I molecules can also provide additional CD8-mediated signaling required for T cell activation under conditions of either subthreshold levels of anti-TCR mAb or subthreshold levels of MHC/Ag complexes. Thus, potential target cells with low Ag/MHC density must provide additional costimulatory signals in order to induce CTL degranulation, and these additional signals can be provided by "bare" MHC class-I
molecules. This is particularly relevant to Burkitt's lymphoma cells and latency type-I EBV transformed B cells in vivo, which lack the costimulatory molecule B7 and have very low levels of MHC class-I molecules on their cell surface (Masucci et al., 1987) (Miyashita et al., 1995). Thus, while Ag/MHC density may directly influence CTL responsiveness, it is equally clear that costimulatory signals also play an important role (Jenkins et al., 1990). Crosslinking of the TCR in the absence of CD28 ligation by B7 stimulates T cell activation followed by apoptosis. Importantly, B7 is expressed only on activated B cells (Clark and Lane, 1991), and is not present on latency type-I EBV infected B cells in-vivo.

Our results demonstrate that the in-vivo activated CD8+ CTL present during primary EBV infection clearly includes a large EBV-specific HLA-restricted component. Recent evidence suggests that the currently accepted understanding of the ultimate fate and function of these in-vivo primed CTL may be incorrect. Traditional methods for generating EBV-specific T cell lines involves in vitro stimulation of memory CTL with the autologous BLCL. A relatively frequent outcome of such efforts is the generation of CTL lines which proliferate in response to BLCL stimulation, but which do not lyse the 51Cr-labeled BLCLs in cytotoxicity assays (Hill et al., 1995). Importantly, superinfection of the autologous BLCL used to stimulate the CTL, with recombinant vaccinia virus encoding EBV nuclear proteins, confers susceptibility to lysis by these CTL. The authors of this particular study have interpreted their findings to suggest that many or all of the "alloreactive" and other "nonspecifically activated" CTL present during acute EBV infection, may after all be EBV-specific but require this additional antigen to demonstrate EBV-specificity in vitro. There is good evidence to support this interpretation which may serve to explain why autologous BLCL do not competitively inhibit lysis mediated by many of the "nonspecifically activated" CD8+ CTL present during acute EBV infection. We believe this interpretation to be correct and support the authors contention that screening for these CTL will likely demonstrate that the majority of activated CD8+ T cells present during acute EBV infection are in fact EBV-specific. Thus, one must remember that the usual procedures used to
screen for EBV-specific CTL would preclude identification of CTL with this effector phenotype, suggesting that published data on EBV-specific CTL should be considered incomplete. This concept is particularly important here, as we have identified EBNA1-specific CTL activity present during acute EBV infection which, with one exception, cannot be demonstrated in the memory CTL response by the usual methods used to screen for memory CTL.

In contrast to the paucity of information on the specificity of viral antigen recognition by activated lymphocytes during primary EBV infection (Strang and Rickinson, 1987) (Strang and Rickinson, 1987), there is an abundance of information on the EBV-specific memory T cell response (Masucci et al., 1993). In fact, in a recent review on cellular immunity to EBV, the author states that "the early nonspecific response to EBV-carrying immunoblasts that characterize the primary infection is followed by the appearance of a persistent, specific T cell immunity" (Masucci and Emberg, 1994). In this regard, Dr. Abul Abbas has suggested that because B7 is such a potent stimulator for CD8+ cells, overexpression of B7 on activated B cells may lead to T-lymphoproliferation of CTL which kill or at least inhibit activated B cells (Abbas, personal communication). While there are clearly many activated CTL of unknown antigen specificity present during acute IM, our data demonstrate that the primary immune response is also EBV-specific, and in many respects this broadly reactive primary CTL response closely reflects the eventual, matured memory CTL response.

In our work we did not evaluate the fine antigen specificity of individual CTL clones, and thus we cannot rule out the possibility that the EBNA1-specific CTL activity we observed with in-vivo activated CTL may represent allospecific CTL, which display cross-reactivity to EBNA1. Many groups (Strang and Rickinson, 1987) (Yang et al., 1989), including ours (Tomkinson et al., 1989), have described the massive stimulation of allospecific CTL during acute IM, and likewise, many have described the simultaneous presence of EBV-specific CTL during primary infection (Strang and Rickinson, 1987). Similar experiments in murine systems have suggested that during acute virus infection, viral peptides presented by self MHC may
look like alloantigen to some CTL, resulting in the activation of low-avidity, virus-specific CTL which cross-react to alloantigen (Nahill and Welsh, 1992). Additionally, allospecific CTL which cross-react with viral antigen may play a role in the lysis of virus-infected cells early during infection, when viral titers are highest, and prior to the expansion of high affinity, virus-specific CTL. That many of the early virus-specific CTL are of low affinity is confirmed by a 50% reduction of virus infected targets by anti-CD8 (Nahill and Welsh, 1993), suggesting low affinity interaction dependent on CD8 accessory molecule interaction. Thus, it is entirely possible that in-vivo-activated allospecific CTL contribute to the control of primary EBV infection, and further that their TCR avidities are likely too low to sustain these cross-reactive cells during the higher avidity memory CTL response. This phenomenon could certainly explain the sequential disappearance of both allospecific and low avidity, virus-specific CTL as the immune response to virus infection subsides. Whether or not our observed EBNA1-specific CTL activity resides in such a low affinity CTL population remains unknown.

**Clinical significance of EBNA-1 expression in malignancies.** EBNA1 is the only viral protein consistently expressed in all EBV associated malignancies, and as yet there has been no accepted/convincing demonstration of EBNA-1-specific CTL (Masucci and Emberg, 1994). Clearly, evasion of any host CD8+ CTL response against EBNA-1 is essential to EBV persistence. The most complete viral evasion mechanism described thus far involves the establishment of true viral latency, typified by HSV and VZV, where infected host cells contain the viral genome, but do not express any viral proteins (Marrack and Kappler, 1994). EBV utilizes a similar, though less complete mechanism of viral escape, whereby EBV establishes a type-I latent state in B cells, characterized by tightly restricted viral gene expression limited to EBNA1. Recent data suggests that the inability to detect EBNA1-specific CTL lies in the inhibition of antigen processing conferred by the internal Gly-Ala repeat of EBNA1 (Levitskaya et al., 1995). The authors suggest that this highly selective
strategy of viral escape would favor the long-term persistence of EBV-infected cells without inducing immunosuppression, thereby allowing for the maintenance of memory responses geared toward suppressing the uncontrolled proliferation of EBV-transformed lymphoblasts. This concept is in keeping with the long accepted view that resistance of cells expressing EBNA1 to CTL-mediated rejection is a critical requirement for EBV persistence, yet it does not sufficiently account for the lack of CTL mediated rejection of EBV positive Hodgkin's lymphoma or NPC cells, which express two highly immunogenic viral proteins (LMP-1 and -2) in addition to EBNA1 (Grasser et al., 1994). Thus, other immune escape mechanisms must exist to account for EBV persistence and the development of BL, NPC and HD in immunocompetent individuals (Frisan et al., 1995).

Evidence against the existence of EBNA-1-specific CTL. The search for EBNA1-specific immunity has generated much scientific interest, and many believe EBNA1 does not contain CTL epitopes. However, the majority of published studies have not been specifically designed to identify EBNA1 CTL epitopes and thus much of this belief is based upon a lack of evidence (Khanna et al., 1992) (Murray et al., 1992). The earliest indication that EBNA1 may be a poor target for CTL recognition came from studies describing the absence of CTL activity against Burkitt's lymphoma cells which expressed EBNA1. Subsequent work in a mouse tumor rejection model demonstrated that unlike LMP1, expression of EBNA1 did not confer immunogenicity to non-immunogenic murine carcinoma lines (Trivedi et al., 1991) (Trivedi et al., 1994). These results have generally been interpreted to imply that EBNA1 would also be non-immunogenic in humans.

Possible explanations for the reported absence of EBNA-1-specific CTL. It is entirely feasible that the reported inability to demonstrate EBNA1-specific CTL activity is due to some unknown technical obstacle, resulting in repeated description of an "in-vitro artifact". For
example, the use of BLCL to restimulate memory CTL likely favors the outgrowth of CTL specific for the immunodominant EBV antigens, of which EBNA1 may not be included.

As mentioned above, recent data suggests the mechanism responsible for the absence of EBNA1-specific CTL may lie in the inhibition of antigen processing conferred by the internal Gly-Ala repeat of EBNA1. The Gly-Ala repeats encompass approximately one third of the EBNA1 protein and the majority of the antibody response to EBNA1 is directed against this repeat sequence. In target cells generated by insertion of the EBNA1 Gly-Ala repeat into the EBNA4 (EBNA3b) gene, target cell lysis by an EBNA4-specific CD8+ CTL clone (epitope 416-424) was reduced by an average of 50% over wildtype EBNA4, but never completely eliminated. Similarly, independent overexpression of EBNA1 did not prevent recognition of Vac-EBNA4 (3b) infected fibroblasts by the CD8+ CTL, but rather reduced lysis by 30-50%. Thus, the Gly-Ala repeat in EBNA1 appears to reduce target cell lysis directed against proteins containing the repeat, but does not significantly affect the recognition of other viral proteins. While this mechanism would likely reduce the potential for EBNA1 directed CTL lysis, the levels of lysis observed here against proteins containing the the Gly-Ala repeat, correlate closely with the levels of EBNA1 mediated lysis detected in our studies (10-25% specific lysis at a MOI of 10:1). Thus, the Gly-Ala repeat of EBNA-1 is likely to be only partially responsible for the lack of identified EBNA-1-specific CTL.

The complexity of events involved in foreign antigen processing and presentation to CD8+ CTLs has provided DNA viruses with ample opportunity to interfere with and prevent viral antigen recognition. A common mechanism employed by several DNA viruses involves a reduction in the synthesis of new MHC class I molecules. The adenovirus Ela product downregulates MHC class-I heavy chain transcription (Paabo et al., 1989), and the herpes simplex virus UL41 protein exerts a more general inhibition of host cell protein synthesis. Along these lines, down-regulation of HLA class-I molecules, by some unknown mechanism, has been proposed as another mechanism of EBNA-1 immune escape in Burkitt's lymphoma cell
lines (Masucci et al., 1987). Similarly, downregulation of cell adhesion molecules LFA-3 and ICAM-1 in EBV-positive Burkitt's lymphomas has been proposed to underlie tumor cell escape from virus-specific T cell surveillance (Gregory et al., 1988). Subsequent work has demonstrated a major role for ICAM-2 in the recognition of BL cells by CTL, with little if any contribution from BL-associated LFA-3 or ICAM-1 (Khanna et al., 1993).

Many viruses have also evolved sophisticated mechanisms to interfere with the assembly and transport of peptide/MHC class-I complexes. For example, inhibition of TAP1/TAP2 transporter gene expression by adenovirus 12 prevents effective antigen presentation by reducing the delivery of peptides to the ER for binding to class-I molecules (Rotem-Yehudar et al., 1994). Similarly, Herpes Simplex virus-1 encodes an immediate-early, cytosolic protein ICP47, which mediates its inhibitory effects by blocking the delivery of antigenic peptides to the site of Ag/MHC assembly, causing the retention and degradation of empty class-I molecules in the endoplasmic reticulum (York et al., 1994) (Hill et al., 1995) (Fruh et al., 1995). Many believe this mechanism may be responsible for the overall paucity of HSV-specific CTLs identified thus far, and may help explain the predominance of CD4+ over CD8+ HSV-specific CTLs observed in vivo. The notion that ICP47 represents an efficient inhibitor of CD8+ T lymphocyte responses in humans, and may therefore foster CD4+ responses, is particularly intriguing given the recent description of a CD4+ EBNA1-specific CTL clone (Khanna et al., 1994). While the effector CTL used in our assays were predominantly CD8+, we did not specifically deplete CD4+ cells and accordingly the EBNA1-specific target cell lysis observed in our primary CTL assays could theoretically reflect CD4+ T-lymphocyte mediated cytotoxicity. The description of this CD4+ EBNA 1-specific CTL clone suggests that future attempts to evaluate EBV-specific CTL should be performed with both NK (CD16) depleted, and CD4+ depleted effector cells, and include cold target competition studies.

Interestingly, inhibition of antigen presentation by ICP47 is species specific as well as cell type specific, as ICP47 does not inhibit antigen presentation in HSV-1 infected murine cells,
and similarly does not prevent antigen presentation in EBV-infected human B cells.

Discrepancies between results derived in humans and mice, such as with ICP47, serve to remind us of the risks inherent in attempting to extrapolate results from work in mice to that of human systems. This issue is poignant here, as already mentioned, the inability to generate EBNA1-specific CTLs in EBNA-1 immunized mice (Trivedi et al., 1991) (Trivedi et al., 1994) has been interpreted to imply that EBNA1 would also be non-immunogenic in humans. The danger in making this assumption is demonstrated by the described disparity in the immunodominant CD8+ CTL response to murine CMV, which differs substantially from CD8+ human CMV response in terms of antigen specificity.

Similar to HSV, both human and murine CMV interfere with MHC class-I expression and CTL recognition. Murine CMV achieves this via expression of an early gene which specifically blocks the transport of peptide-charged MHC class I complexes to the cell surface (Hengel et al., 1994). Similarly, human CMV evades T lymphocyte recognition by down-regulating MHC class-I expression via their retention and rapid degradation in the endoplasmic reticulum (Beersma et al., 1993). Despite these adverse effects on the class-I antigen presentation pathway, CD8+ CTL play a crucial role in the immune response to both HSV and CMV, highlighting the adaptability of the human immune system in overcoming complex obstacles presented by viruses. Interestingly, the effect of this CMV early protein on antigen presentation in murine cells can be overcome by IFN-γ, which mediates a 25 fold increase in the synthesis of MHC class I molecules.

Mutation of potential EBNA1 epitopes is unlikely to play a role in explaining the absence of EBNA1-specific memory CTL. While mutation of individual antigenic epitopes recognized by CD8+ CTL have been reported for EBV, it has only occurred within a HLA-A11 restricted EBNA-3b epitope, and confined to a geographic area where A11 is a very common allele among the population (deCampos-Lima et al., 1993). In general, the EBV genome is highly stable and antigenic drift is not considered to be a common nor significant factor in
immune evasion by EBV.

Secreted viral proteins can also interfere with host immune responses. For example, EBV encodes an immediate early gene BCRF1, which is homologous to human IL-10 (Hsu et al., 1990), and has been shown to stimulate B cell growth, downregulate expression of the cell surface B7 costimulatory molecule (Ding et al., 1993) (Schandene et al., 1994) (Willems et al., 1994), and inhibit the activation of CTLs by suppressing macrophage activation and Th1 lymphokine production (deWaal-Malefyt et al., 1991). IL-10 has also been shown to inhibit IL-2 secretion by T cell clones and purified peripheral blood T cells following stimulation with anti-CD3 mAb plus fibroblast associated B7 (deWaal-Malefyt et al., 1993). Thus, IL-10 exerts its effects on the immune response by both reducing the expression of B7 on APCs and by inhibiting some B7-mediated T cell activation events. However, IL-10 does not inhibit the reactivation of memory CTL responses to BLCLs in-vitro (Bejarano et al., 1992), suggesting that the reported expression of this gene during EBV lytic infection may target this mode of escape to the replicative phase rather than the latent phase of EBV infection.

Lastly, a more general and potentially very important mechanism of immune evasion by EBV-infected cells expressing EBNA1 involves the poor immunogenicity of resting B cells, which do not express the adhesion or costimulatory molecules necessary for the effective triggering of EBNA1-specific CTL (Miyashita et al., 1995). Importantly, rather than activating resting T cells, resting B cells tolerize them to specific antigens (Parker, 1993) (Clark and Ledbetter, 1994). Consequently, crosslinking of CD28 on T cells by B cell-associated B7 prevents specific unresponsiveness or apoptosis that would otherwise occur following stimulation of the T cell receptor (Harding et al., 1992). CD28 is expressed on >80% of resting peripheral T cells (Linsley et al., 1990), and blocking CD28/B7 interaction during antigen-specific stimulation of T cells results in a state of antigen-specific T cell unresponsiveness, which can prolong cardiac allograft survival (Lin et al., 1993), induce tissue graft tolerance in a pancreatic islet cell graft model (Lenschow et al., 1992), and inhibit T cell-dependent antigen-
specific immune responses in vivo (Linsley et al., 1992).

**Our results demonstrating EBNA-1 CTL activity.** Evaluation of EBV-specific CTL responses stimulated during primary EBV infection in 35 acute IM patients revealed EBNA-1-specific responses in 7 patients (table 7). Effector cells, prepared from in-vivo primed PBMC, were rosette selected with SRBC and depleted of NK cells by anti-CD16 mAb plus complement. Effector cells prepared in this manner were analyzed by FACS analysis and consisted of predominantly CD3+/CD8+/HLA-DR+/CD11b+/CD4-/CD16-. Additionally, the majority of target cell lysis mediated by these effector cells could be blocked by anti-CD8 mAb and/or anti-class-I mAb, while anti-CD4 mAb had little or no effect. These findings are consistent with the presence of a population of EBNA-1-specific CD8+ CTL during primary EBV infection, which likely bear low affinity TCR and are almost uniformly absent from the higher-affinity EBV-specific memory CTL population.

**Integration of our results within context of evidence or hypotheses in literature.** Recent evidence suggests that small, high-buoyant-density peripheral blood B cells represent the EBV reservoir in healthy virus carriers. These B cells express only EBNA1 in the absence of other known viral products (Chen et al., 1995), and are believed to maintain stable levels of EBNA1-specific antibodies throughout persistent infection. More importantly, we now have good reason to believe that the unique regions of EBNA1 represent potential target epitopes for CTL responses, since they encode several peptides capable of inducing expression of MHC class I molecules in murine and human cell lines with defective transporters of antigenic peptides (Stuber et al., 1995). Thus, published speculation that EBNA1 does not contain peptides capable of binding to MHC class-I molecules should be discarded.

The precise mechanism responsible for the apparent disappearance of EBNA 1-specific activity following the acute phase of EBV infection remains unknown, however this
observation is not unique to EBV. During acute infectious mononucleosis, as with many primary virus infections, the host immune response must be regulated so as to minimize bystander damage caused by over-exuberant responses. The practical implications of this concept mean that variables influencing precursor frequency of memory cells must be well regulated. In order to understand the factors influencing the generation of immunologic memory cells, one must consider certain aspects of the primary response, particularly the fate of the large number of acutely activated lymphocytes. Under certain circumstances clonal stimulation can lead to tolerance, or conversely, to immunologic memory. In an elegant set of experiments complete antigen-specific CTL exhaustion was demonstrated by showing that when LCMV-specific TCR transgenic T cells are transferred to LCMV-infected mice, the donor T cells undergo intense proliferation followed by complete elimination of the donor T cells, leaving the mice functionally tolerant without clearance of the virus (Moskophidis et al., 1993) (Moskophidis et al., 1994). Importantly, complete elimination of T cells only occurred when cells were exposed to high doses of virus. Lower doses of virus produced a more intense CD8+ proliferative response, resulting in rapid virus clearance. Here, the authors suggest that virus may have persisted as a result of such complete induction/exhaustion of specific antiviral CD8+ CTL that the virus was not eliminated, and further that partially induced (protective) immunity and complete CTL exhaustion (peripheral tolerance) represent quantitatively different points on the immunity scale. Similar examples of immunity induced extrathymic tolerance via mature T cell elimination have also been described (Webb et al., 1990). Accordingly, we observed the disappearance of EBNA1-specific CTL activity following acute EBV infection, which may reflect either the induction of anergy or the peripheral deletion of EBNA1-specific CTL.

The cell surface concentration of antigen presented by APCs can also directly influence the outcome of TCR encounter with antigen, as was recently demonstrated in a transgenic mouse model (Ferber et al., 1994). In this case, expression of minute amounts of MHC antigen on hepatocytes induced tolerance by partial downregulation of TCR on self-reactive CD8+ cells.
Importantly, subsequent contact of tolerant T cells with high doses of MHC resulted in complete TCR downregulation, suggesting that tolerant T cells are susceptible to further tolerogenic signals, including high doses of antigen/MHC complexes. Thus, if EBNA1-specific CTL are present during the primary immune response, and partially tolerized following contact with B7-deficient type-I EBV-transformed-B cells in vivo, then the above mouse model data would suggest that subsequent restimulation with high doses of BLCL-derived antigen in vitro may drive the partially tolerant T cells further into a state of complete EBNA1-specific tolerance.

Another example of specific T-cell tolerance preceeded by a primary response was recently described by the Benacerraf lab (Vidard et al., 1994), where they detected an early primary T-cell response in mice tolerized with Ova, prior to the establishment of Ova T-cell tolerance. The authors of this work suggest that in vivo unresponsiveness can result from the same experimental procedures used to grow T-cell lines in vitro, whereby, unless the cells are allowed to rest after each stimulation, over-stimulation leads to unresponsiveness. They further suggest that when a primary response preceeds secondary unresponsiveness, anergy cannot be invoked to explain the tolerance, as strictly speaking, anergy is defined as the recognition of antigen by cells lacking costimulatory molecules. Accordingly, the correct term for tolerance described here is refractoriness, which describes the state of activated T cells that have abandoned some or all of their functional activities. The fact that EBV persists for life in type-I transformed B cells in the peripheral blood of all healthy EBV-seropositive individuals, suggests it may serve as a "constant source of EBNA-1 over stimulation", leading to EBNA-1 unresponsiveness. In our opinion, the persistent presentation of EBNA-1 by quiescent B cells, lacking the B7 (CD80) molecule, is unlikely to result in overstimulation of CTL. However, the impressive T-lymphocytosis present during acute IM suggests that overstimulation would be more likely to occur during primary EBV infection as opposed to convalescence.

Similar to the discrepancy between primary vs memory CTL responses to EBV described in this dissertation, a group from Louisiana has reported preliminary evidence describing a
HSV-1 epitope-specific CTL population present at high frequency during primary infection which is absent from the memory response (Nugent and Jennings, 1994).

**Importance of identifying EBNA-1 CTL antigens for vaccine development.** Latent EBV infection represents an important model to study vaccines for human cancers as vaccine development for virus-associated tumors is strategically simpler than attempting to identify protective tumor antigens in tumors of unknown etiology.

The development of successful vaccines for human virus infections is based upon an understanding of the effector cells and immune mechanisms required to provide the host with a protective response to acute infection and which promote memory responses to provide protection during persistent viral infections. Additionally, there is now increasing interest in the use of CTLs to destroy tumor cells in vivo. In this context, the identification of immunogenic EBV antigens offers the potential for designing vaccine based immunotherapy against EBV associated malignancies. The significance of identifying EBV CTL epitopes is underscored by the description of EBV peptide epitopes which sensitize Burkitt's lymphoma cells to lysis by EBV-specific CTL (Khanna et al., 1993). However, if CTL therapy is to be fully exploited in the treatment of the diverse array of EBV associated malignancies, then we must first strive to understand why a given individuals CTL response is unable to destroy tumor cells. For example, is there a deficiency of relevant CTLs, or has the virus modified the tumor cells in such a way that relevant antigens now deliver inhibitory signals to inactivate potentially protective CTLs.

The primary benefit of an EBV vaccine for Western societies would be to prevent the symptoms of acute infectious mononucleosis. This very realistic goal does not require that the vaccine induce sterile immunity, but rather produce a population of memory cells which would limit virus replication and spread during primary infection. An equally important, though currently less achievable goal of an EBV vaccine, would be to prevent the establishment of
latent EBV infection, since the majority of EBV associated malignancies develop several years after primary infection. The continued addition of newly recognized EBV-associated tumors including gastric carcinoma and adult T cell leukemia lymphoma this year, suggest the list is not complete. Accordingly, patterns of EBV gene expression in association with diseased tissue, and specific T cell responses to EBV antigens in these patients will be needed.

Our analysis of the immunodominant T cell response during primary EBV infection suggests that an effective EBV vaccine necessarily include at minimum, all three proteins in the EBNA-3 family, and also the lytic cycle protein gp350. Our work has demonstrated that greater than 90% of patients with acute IM have CTL which lyse target cells expressing one or more of the EBNA3 proteins, suggesting that such a vaccine would be expected to protect the majority of individuals. While only 20% of acute IM patient CTL lyse targets expressing gp350, we feel it should be included in any potential EBV vaccine, based in part on some compelling in-vivo evidence generated by other groups. Immunization of marmosets with mAb affinity-column purified gp350 does not protect against EBV in spite of high virus-neutralizing titers (Epstein et al., 1986), suggesting that in addition to neutralizing antibody, other immune mediators, such as CTL, are required to prevent the development of EBV-associated lymphomas. In order to overcome some of the observed variations in immunity induced by various gp350 antigen preparations, the gp350 gene was inserted into a vaccinia virus vector to allow for the homogeneous expression and post-translational processing of this glycoprotein. Curiously, the gp350 vaccinia construct did not induce EBV-neutralizing antibody but did protect cottontop marmosets against a lymphomagenic dose of transforming EBV (Morgan et al., 1988). This unexpected finding clearly suggested the need to examine the cell mediated immune response against this major EBV glycoprotein. Thus, it would be prudent to more completely define the primary CTL response to gp350 in acute IM patients. The potential benefits of including EBNA1 in an EBV vaccine remain uncertain, and may require removal of the Gly-Ala repeat, but will surely require more in vivo studies.
Convincing evidence that minor CTL reactivities, in addition to the immunodominant CTL response, may play an important role in the immune protection against viruses was recently provided by Oldstone et al (Lewicki et al., 1995). When the viral genomic sequence encoding the immunodominant CTL epitopes of LCMV is mutated, the CTL response is shifted toward novel virus epitopes not previously recognized. The authors have suggested that CTL epitopes typically form a hierarchy, in which responses to "weak" epitopes are suppressed in the presence of "stronger" epitopes. Thus, mutation of the dominant epitopes may be of limited advantage to a virus, since the host can then mount a protective response directed against the weaker T cell epitopes.

The rational design of an EBV vaccine must also incorporate new evidence that EBV proteins other than latent genes may provide significant CTL epitopes. Cytotoxic T lymphocytes specific for the immediate-early transactivator ZTA (BZLF1) have recently been described (Bogedain et al., 1995). This finding is particularly important as CTL directed against this protein have the potential to eliminate EBV-latently infected cells at the earliest stage of switch to lytic cycle replication. Oldstone et al. have suggested just such an early gene-based vaccine approach to prevent persistent viral infection (Oldstone et al., 1993). Evidence suggests that EBV transformed B cells in vivo, which phenotypically represent type-1 latency, can undergo ZTA-mediated switch from latency to virus replication, in the absence of other latent gene expression (Rowe et al., 1992). Additionally, ZTA has been detected in small numbers of tumor cells in EBV-associated Hodgkin's disease, EBV-associated non-Hodgkin's lymphomas in AIDS patients, and posttransplant lymphoproliferative disease (Pallesen et al., 1991). Accordingly, ZTA-specific CTL activity should be sought in a broader base of patients and the ZTA protein should be carefully considered for inclusion in any EBV vaccine.

The significance of B7 costimulation in tumor immunity is underscored by the demonstration that nonimmunogenic tumors become immunogenic following transfection mediated expression of the B7 gene (Chen et al., 1992) (Townsend and Allison, 1993). In fact,
one group has suggested that continued TCR stimulation by foreign antigens presented on or released from tumors in the absence of adequate costimulation, by molecules such as B7, will suppress tumor immunity (Hellstrom et al., 1993). Thus, this phenomenon may account for the absence of reported EBNA1-specific memory CTL and may also explain why LMP1+/LMP2+ NPC tumor cells can grow in immunocompetent hosts. This concept is potentially important, since EBV expresses an IL-10 like molecule which has been demonstrated to prevent B7 up-regulation on IFN-γ-activated human monocyes (Willems et al., 1994), and murine macrophages (Ding et al., 1993). Importantly, the majority of human tumors do not express B7, and mice which have rejected B7-transfected tumors acquire the capability to mediate rejection against wildtype tumors lacking B7. Moreover, the newly acquired tumor-specific response is mediated by CD8+ CTLs (Harding and Allison, 1993). Thus, B7-mediated CD28 costimulation provides an attractive approach to enhance tumor immunity. Unfortunately, transfer of B7 into nonimmunogenic lymphomas does not always induce an effective T cell response (Chen et al., 1994). This is not completely unexpected, as naive T cells respond best to costimulatory molecules when expressed on the same cell as the TCR ligand (Liu and Janeway, 1992), and only cells which express costimulatory molecules can effectively activate naive T cells. This would suggest that when T cells encounter antigen on B7 negative cells, they are likely to be rendered anergic, even if B7+ cells are also present. Evidence that T cell tolerance to EBNA1 could be maintained in vivo, in the presence of B7+ BLCLs, comes from the demonstration that T cell tolerance to myelin basic protein is maintained in the presence of costimulation provided by transfectants which expresses high levels of B7 (LaSalle et al., 1992). This phenomenon is likely very important in maintaining tolerance to other self-tissues, as it allows active costimulatory cells to coexist with costimulator-negative cells expressing self-antigens not found in the thymus, where strongly self-reactive T lymphocytes are eliminated (Nossal, 1994). If this concept is correct, then one would predict that CD8+ EBNA1-specific CTL would be rendered anergic by the in-vivo pool of B7− EBV-infected B cells present during convalescence.
(Miyashita et al., 1995), and maintain tolerance to the phenotypically activated, EBV-transformed lymphoblasts expressing high levels of B7. These findings have potential therapeutic implications for B7+ EBV-associated tumors and may provide an approach to achieving sterile immunity against EBV (Roitt, 1994).

As recently suggested by Thorley-Lawson et al., in order to construct a relevant model for EBV latency in vivo, it is necessary to know whether EBV-infected cells are lymphoblastoid or resting. In fact, EBV-infected cells in the peripheral blood are predominantly (>95%) CD19+/CD23-/CD80- (B7-). Accordingly, when TCR engagement is accompanied by a costimulatory signal, a T cell will proliferate and differentiate, whereas the absence of costimulation usually results in anergy or cell death. Thus, encounter with antigen can lead quite distinct outcomes, proliferation and differentiation vs inactivation or death, depending upon the nature of costimulatory signals.

**MHC class-I restriction elements.** The recognition of viral peptides by CD8+ CTL is restricted through MHC class-I molecules on infected cells. Additionally, a given peptide typically associates with certain MHC molecules in accordance with amino acid sequence of the peptide and the properties of the MHC peptide binding site. In this regard, many EBV peptide/HLA class-I molecule pairs have been identified, which allow comparison with our data. Of the nine patients who lysed EBNA2 expressing targets, 7 (78%) carry MHC class-I alleles previously demonstrated to be restriction elements for EBNA2 (Masucci et al., 1993) (Lee, 1994). Other alleles present in high frequency of EBNA2 specific CTL include A1 (44%), and B40 (33%). Similarly, 18/22 patients (82%) recognizing EBNA3A share previously identified class-I restriction elements. Interestingly, two of the other 4 patients carried the A11 allele, and all 4 patients carrying the A11 allele recognized EBNA3A. Importantly, A11 is particularly rare among the general U.S. population (3%). With regard to EBNA-3B, only 6/20 (30%) IM patients carry MHC class-I alleles previously described as restriction elements for
EBNA-3B (A11 and B8). However, all patients carrying the A11 allele recognized target cells expressing EBNA-3B. Our data suggests that other possible restriction elements include A2, A3 and B40 which were present in 13/14 (93%) of the A11-/B8- patients who recognized EBNA-3B. Interestingly, subsequent to our findings, A2 has been described as a restriction element for EBNA-3B, carried by 6 of our patients, bringing the percent of patients with known restriction elements to 12/20 (60%). Of the remaining 8 patients, seven (88%) carry either A3 or B40 alleles. In patients who recognized EBNA-3C, 27/29 (93%) carried MHC alleles which have been reported to serve as restriction elements for EBNA-3C. Amazingly, 11 of 13 patients (85%) with the A2 allele recognize EBNA-3C, and 8/10 (80%) of HLA-B7 patients recognize EBNA-3C, suggesting a preference for EBNA-3C epitopes by the A2 and B7 alleles. EBNA-LP restriction elements have not been described, although our data did show a higher than expected prevalence of the B7, B13 and B39 alleles. Four of five (80%) patients recognizing LMP1 carry previously defined restriction elements A24 and B40, and significantly, 60% also carry the A28 allele. In fact, virtually 100% of patients recognizing LMP1 carried either the A28 or B40 alleles, or both. Three of five (60%) of patients carry restriction elements described for LMP-2 (A2) while no other alleles were obviously dominant. Class-I restriction elements for gp350 have not been described, however, of the 7 patients in our study who recognized gp350, 5 were HLA-typed, 3 of which (60%) carried the A2 allele. Similar to gp350, no class-I restriction elements for EBNA1 have been described.
### Summary of Previously Defined MHC Class-I Restriction Elements for EBV Epitopes

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<td>+</td>
<td>+</td>
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<td>-</td>
</tr>
</tbody>
</table>

- : No CTL response at clonal or polyclonal level in any donor.
+ : Weak CTL response in some donors.
++ : Strong CTL response in polyclonal culture and rare clones in some donors.
+++ : Strong CTL response at polyclonal and clonal level in all donors tested.

Phenotype of effector cells. The hallmark of immunologic memory is the ability of lymphocytes to respond more quickly, specifically, and with greater magnitude upon second exposure to a given antigen. Accordingly, the goal of vaccination is to prime the immune system to make anamnestic responses against infectious pathogens. Unlike memory B cells which undergo somatic hypermutation, memory T cells do not participate in "affinity maturation" of their TCRs (Vitetta et al., 1991). Thus, other less specific means of distinguishing memory from naive T cells have evolved, based primarily upon the differential expression of cell surface molecules involved in T cell adhesion and activation. CD45 is one well studied surface molecule used to distinguish memory and naive T cells in humans. The CD45RA molecule on naive T cells (200-220 kDa) undergoes alternative RNA splicing to produce the lower molecular weight form (CD45RO) present on memory T cells (Akbar et al., 1993). This distinction is supported by the finding that human CD8+/CD45RO+ memory CTL have less stringent requirements for in vitro activation than naive CD8+/CD45RA+ T cells (DeJong et al., 1991). To complicate the simple scheme of CD45RA and CD45RO marking naive and memory T cells respectively, reversal of CD45R isoform switching has been demonstrated in CD8+ T cell (Fuji et al., 1992). Thus, with the possible exception that CD8+/CD45RA- T cells are primed, there are no other generally accepted phenotypic cell surface markers that distinguish memory from virgin T cells. In fact, most markers associated with memory are activation markers (Akbar et al., 1991), or adhesion molecules (Cerottoni and McDonald, 1989) (McFarland et al., 1992). The significance of altered adhesion molecule expression on memory T cells can be appreciated by understanding that this process increases the affinity of T cells for antigen presenting cells. By analogy with memory B cells, which increase antigen binding affinity through somatic mutation of their receptors, memory T cells may alter adhesion molecules to accomplish the same goal of higher affinity. In this context, it is important to point out that some authors have suggested that memory T cell responses in-vivo require periodic restimulation with the original priming antigen for maintenance, and thus may actually represent sub-optimally stimulated effector cells (Gray
and Matzinger, 1991). However, other groups have argued that re-exposure to virus or antigen is not required for the maintenance of memory CD8+ T cells (Ahmed, 1992) (Mullbacher, 1994).

β2-integrins such as Mac-1 (CD11b) and LFA-1 are extremely versatile adhesion molecules, which both bind ICAM-1 and can be rapidly modulated by the cells expressing them. Importantly, some very convincing data suggests that CD11b represents a marker for CD8+ cytotoxic T cell activation and memory during virus infection (McFarland et al., 1992). The authors have shown that CD11b is present in high levels (~50% of CD8+ cells) on activated LCMV-specific CTL during acute infection, and that depletion of CD11b+ T cells results in the inability to mediate in vitro secondary LCMV-specific responses. Furthermore, virtually all of the virus-specific killing was mediated by the CD8+/CD11b+ cell population. Our results support these findings and show that while CD8+/CD11b+ T cells comprise only 5% of healthy donor peripheral blood lymphocytes, levels of CD8+/CD11b+ T cells in peripheral circulation rise by 8-12 fold (40-60%) during the peak of the EBV-specific CTL response. In fact, 50-85% of CD8+ T cells coexpress CD11b during primary infection with EBV. In contrast to the LCMV model, where expression of CD11b by CD8+ splenocytes declined to near naive levels by day 19-20 p.i., the percentage of CD8+/CD11b+ T cells in the peripheral blood of acute IM patients remained markedly elevated at 6 weeks p.i., and had only declined to near naive levels by 6 months. The longevity of CD11b expression by CD8 immune lymphocytes also differed from the T cell activation marker HLA-DR, which peaked during the acute phase of primary EBV infection and was reduced by 50% at 6 weeks p.i. In our hands, CD11b is quickly lost during in vitro culture (4-5 days), and thus, as has already been suggested, CD11b may be the single best in-vivo marker to identify memory CTL. The following model depicts a scheme for identifying memory and naive T cells bearing various cell surface markers.
Development of T cell memory

CD45RA+ T cells
- Respond poorly to recall Ag in-vitro
- Present at birth, decrease with age
- Require professional APC for activation

CD45RO+ T cells
- Respond well to recall Ag in-vitro
- Low #s at birth, increase with age
- High expression of adhesion molecules

Model of T cell memory: based upon work of Mueller, Jenkins and Schwartz. Antigen specific T cells expand prior to entering the state of anergy, which occurs about one week post antigen stimulation. The reason certain signals induce anergy rather than an effector response and memory generation is unclear, but the triggering of T cells in the absence of costimulator signals is believed to contribute substantially to the development of anergy.

The function of human CD11b (Mo-1) remains unknown, however, it is interesting that the adhesiveness of murine CD11b (Mac-1) can be increased by activation of neutrophils with N-formyl-peptide, and expression can be increased 10 fold by specific chemoattractants. Changes in Mac-1 adhesiveness are thought to be mediated by conformational changes induced during cellular activation, suggested by changes in mAb reactivity with Mac-1 following activation (Diamond and Springer, 1993). Similar affinity measurements of the cell surface integrin LFA-1 have recently demonstrated that cellular activation increases the affinity of a subpopulation of LFA-1 molecules for ICAM-1 by approximately 200 fold (Lollo et al., 1994).

The importance of integrin/adhesion molecule expression for CTL function is highlighted by work demonstrating that in vivo cytotoxicity of CD8+ T clones correlates most closely with their levels of adhesion molecule expression (Rodrigues et al., 1992). In fact, antimalarial protective and nonprotective CTL clones do not differ in their fine epitope specificity or pattern of lymphokine production, but rather in their levels of CD44 and VLA-4 expression. Not surprisingly, the clones also differ in their adhesive properties, and while cross-linking of CD44 can induce LFA-1-mediated aggregation of protective clones, the nonprotective clones fail to aggregate.

Thus, it would not be unreasonable to propose that CD11b may function as an adhesion molecule to strengthen cell-cell interactions, and it may be informative to determine the relative contribution of CD11b to CD8 CTL/target cell avidity, possibly through the use anti-CD11b mAb. Accordingly, one can only speculate on the possible effects of CD11b loss during in-vitro CTL culture, and any potential significance to target cell lysis. However, our current understanding of CD11b suggests that anti-CD11b depletion studies would likely confirm the important role of CD8+/CD11b+ CTL in the long-term immune surveillance against EBV.

In vitro growth of EBNA-1 specific CTL. It is our opinion that many investigators have prematurely come to the incorrect conclusion that the EBNA1 protein contains no CTL epitopes.
This conclusion is based primarily upon data generated with CTL restimulated in-vitro using the autologous BLCL. Current understanding of antigen recognition by cytotoxic T cells entails a multimolecular interaction involving the TCR, antigen, MHC molecule, CD8, B7, CD28 and an ever growing list of adhesion and activation molecules, all bathed in a plethora of lymphokines. We know that antigen recognition by TCR does not always result in T cell activation. In this sense, it is antigen context (ie. costimulation) which regulates the threshold for discriminating self vs foreign antigens. Thus, given that EBV has evolved to establish a dynamic equilibrium consisting of multiple phenotypic forms of latency, each of which differentially affects B cell phenotype, should we not expect multiple outcomes from antigen recognition? Similarly, should we expect in vitro stimulated CTL, exposed only to BLCL cells expressing high levels of activation and adhesion molecules, to recognize an antigen, which in vivo, is expressed in quiescent B cells lacking all adhesion molecules and all but a few MHC class I molecules? While stimulation of EBV-specific CTL with BLCLs has the potential to provide valuable information on the immune recognition of phenotypically similar EBV-positive immunoblastic lymphomas, one should not expect this to accurately represent T cell recognition of type-I EBV-transformed B cells.

Shortly following our original description of EBNA1-specific CTL present during acute IM (Beaulieu and Sullivan, 1994), a second group described a CD4+ CTL clone which recognized the EBNA1 antigen (Khanna et al., 1994). The authors were seeking to specifically identify EBNA1 epitopes, and discovered a CD4+ CTL clone which recognized targets coated (0.1 μg/ml) with a class-II restricted (HLA DR1) peptide from EBNA1. The clone was however unable to lyse EBV infected BLCLs and Vac-EBNA1 infected B cells. Precursor frequency analysis demonstrated the presence of significant numbers of memory T cells specific for this EBNA1 epitope. In our analysis of 32 convalescent IM patients, we identified only one EBNA1-specific memory CTL response, which we were unable to reproduce due to a lack of additional blood samples.
We were also unsuccessful in our attempts to selectively stimulate and expand EBNA1-specific CTL from the peripheral blood of EBV immune donors using aldehyde-fixed APCs. While stimulation of acute IM patient (Levro) PBMC with Vac-EBNA1 initially increased EBNA1-specific lysis by 12.5%, subsequent stimulation favored a Vaccinia-specific response. Curiously, this vaccinia-specific CTL response was observed in several vaccinia naive patients. Cold-target competition assays confirmed the vaccinia specificity, and FACS analysis of the effector cells in one patient demonstrated a predominance of γδ T cells. Our data do not allow us to determine the phenotype of cells responsible for the EBNA-1-specific activity initially present in this culture, and thus one might consider implicating the γδ+ T cells.

In this regard, γδ+ T cells are often detected at sites of viral infection and are believed to participate in host defense (Born et al., 1991). In contrast to αβ+ T cells, which are highly specific effector cells with enormous diversity for antigen recognition, γδ+ T cells represent effector cells with limited diversity for antigen recognition. Many γδ+ T cells recognize targets expressing heat shock proteins, which includes a broad array of cells, including virus infected cells. However, target cell recognition by γδ+ T cells is usually not restricted by classical MHC antigens (Sciammas et al., 1994), and anti-class-I mAb failed to block the γδ-mediated lysis.

One possible explanation for our results comes from the finding that various costimulatory molecules have different sensitivities to aldehyde fixation (Jenkins and Schwartz, 1987). While B7.2 costimulation is significantly eliminated following fixation, B7 is apparently relatively insensitive (Freeman et al., 1991) (Freeman et al., 1993). Additionally, ICAM-1 costimulation is eliminated by paraformaldehyde fixation. Accordingly, the costimulatory capacity of native APCs is significantly greater than that of chemically fixed APCs and should be accounted for in the interpretation of results generated using fixed APCs (Moreno and Lipsky, 1986). Importantly, Harding and Allison have recently shown that anti-CD28 mAb can provide the necessary costimulatory signal to CD8+ T cells, when the costimulator capacity of stimulator cells is destroyed by aldehyde fixation (Harding
and Allison, 1993). This indicates that while fixation destroys the costimulatory capacity of APC, it does not affect the integrity of the antigen-specific signal (Harding et al., 1992). Thus, perhaps the addition of anti-CD28 mAb to our EBNA1-expressing aldehyde-fixed APCs may have altered the outcome in our attempt to restimulate EBNA1-specific CTL in-vitro.

That target cell phenotype influences CTL recognition was nicely demonstrated by measuring the differentiation dependent sensitivity of human B-cell-derived lines to MHC-restricted CTLs (Torsteinsdottir et al., 1986). The sensitivity of in-vitro EBV-infected Burkitt lymphoma lines showed a direct correlation with cell surface phenotype. EBV infected BL lines which acquired BLCL-like characteristics became sensitive to CTL, while BL which retained their original BL phenotype remained insensitive to CTL lysis. This work set a precedent for establishing a link between BL cell phenotype and the accompanying pattern of EBV latent gene expression (Rowe et al., 1987), and also applies to normal B cells (Rowe et al., 1992). Importantly, this same group also described experiments in which early passage EBV+ BL lines were not lysed by MHC-matched EBV-specific CTL. The authors concluded that the finding could not be explained by altered expression of HLA class I antigens, but in several cases did correlate with altered latent gene expression. However, reduced latent gene expression could not explain all cases of BL immune escape, as several BL lines which expressed all latent genes remained insensitive to EBV-specific CTL, likely due to reduced adhesion molecule expression.

The relative contribution of low adhesion molecule expression to immune evasion by BL cells was evaluated in a peptide sensitization assay involving a phenotypically diverse group of BL cell lines (Khanna et al., 1993). Despite reduced levels of the adhesion molecules LFA-1, LFA-3, and/or ICAM-1, peptide-sensitized BL cells were recognized and lysed by EBV-specific CTL. This data suggests that when sufficient antigenic peptide/MHC complexes exist, the reduced expression of adhesion molecules alone will not account for the lack of BL cell lysis by EBV-specific memory CTL. Furthermore, mAb blocking experiments designed to evaluate the
individual contribution of various adhesion molecules in CTL recognition suggested that EBV-specific memory CTL recognize BLCL and Burkitt's lymphoma cells differently. While EBV-specific CTL recognition of BLCLs was dependent on LFA-3/CD2 interaction, CTL recognition of peptide-sensitized BL cells was absolutely dependent on LFA-1/ICAM-2 interaction, with little if any contribution from the LFA-3/CD2 pathway. Importantly, all of the BL cell lines evaluated in this study consistently expressed high levels of cell surface ICAM-2. Thus, downregulation of LFA-1, LFA-3 or ICAM-1 on BL cells does not appear to provide an absolute barrier to tumor cell lysis by virus-specific CTL, though it may prevent recognition of EBV transformed B cells in-vivo with a latency type-1 phenotype.
Table 1. Summary of Patient Information:

<table>
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<tr>
<th>Donor Name</th>
<th>Date of Birth</th>
<th>Infecting EBV Strain</th>
<th>Stage of Infection</th>
<th>MHC Class-I Haplotypes</th>
</tr>
</thead>
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<td>n.d.</td>
<td>Convalescent</td>
<td>A26, A31, B35, B38</td>
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<tr>
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<td>Acute IM</td>
<td>A1, A28, B8, B40</td>
</tr>
<tr>
<td>Arach</td>
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<td>n.d.</td>
<td>Acute IM</td>
<td>A3, A24, B7, B35</td>
</tr>
<tr>
<td>Aumre</td>
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<td>n.d.</td>
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<td>A2, A30, B27, B40</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
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* n. d. = not determined.
Table 2. Established Cell Lines and Corresponding MHC Class-I Haplotypes.

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<th>Cell Line/Origin</th>
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</tr>
<tr>
<td>B95-8</td>
<td>Marmoset B cell, (EBV type-1 producer)</td>
<td>ATCC #: CRL 1612</td>
</tr>
<tr>
<td>Jijoye</td>
<td>Burkitt lymphoma (Hu)</td>
<td>ATCC #: CCL 87</td>
</tr>
<tr>
<td>Namalwa</td>
<td>Burkitt lymphoma (Hu)</td>
<td>ATCC #: CRL 1432</td>
</tr>
<tr>
<td>K562</td>
<td>Human CML line</td>
<td>ATCC #: CCL 243</td>
</tr>
<tr>
<td></td>
<td>EBNA (-), Class-I (-)</td>
<td>EBNA (-), Class-I (-)</td>
</tr>
</tbody>
</table>
Table 2. cont’d.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cell Line/Origin</th>
<th>Class-I Haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV-1</td>
<td>Kidney, fibroblast like (African green monkey)</td>
<td>ATCC #: CCL 70</td>
</tr>
<tr>
<td>MT-2</td>
<td>T-lymphoma, HTLV-1 producer</td>
<td>ATCC #: TIB 162</td>
</tr>
<tr>
<td>ALIAR-MT2</td>
<td>HTLV1 transformed T cell</td>
<td>A26, A31, B35, B38, C4</td>
</tr>
<tr>
<td>BEABR-MT2</td>
<td>HTLV1 transformed T cell</td>
<td>A2, A9, B17, B27, C6</td>
</tr>
<tr>
<td>CROMA-MT2</td>
<td>HTLV1 transformed T cell</td>
<td>A3, A9, B7, B62, Cw3</td>
</tr>
<tr>
<td>HESRU-MT2</td>
<td>HTLV1 transformed T cell</td>
<td>A1, A11, B8, B55, C3, C7</td>
</tr>
<tr>
<td>SULJO-MT2</td>
<td>HTLV1 transformed T cell</td>
<td>A29, A32, B44, Bw60</td>
</tr>
<tr>
<td>TOMBL-Fib</td>
<td>Primary human fibroblast line</td>
<td>A1, A2, B49, Bw52</td>
</tr>
<tr>
<td>SULJO-Fib</td>
<td>Primary human fibroblast line</td>
<td>A29, A32, B44, Bw60</td>
</tr>
<tr>
<td>DELSA-Fib</td>
<td>Primary human fibroblast line</td>
<td>A11, A34, B44, B62, C7</td>
</tr>
<tr>
<td>BLABR-Fib</td>
<td>Primary human fibroblast line</td>
<td>A1, A28, B27, B60, C1, C3</td>
</tr>
<tr>
<td>HUNCA-Fib</td>
<td>Primary human fibroblast line</td>
<td>A2, A28, B27, B53, C1, C7</td>
</tr>
<tr>
<td>BEABR-Fib</td>
<td>Primary human fibroblast line</td>
<td>A2, A9, B17, B27, Cw6</td>
</tr>
<tr>
<td>PIKCH-Fib</td>
<td>Primary human fibroblast line</td>
<td>A25, B38, B53, Cw4</td>
</tr>
</tbody>
</table>

HLA-typing was performed by the HLA typing laboratory at the University of Massachusetts Medical Center.
Table 3. Specifications of recombinant vaccinia virus constructs.

<table>
<thead>
<tr>
<th>Vaccinia Construct</th>
<th>Open Reading Frame</th>
<th>Size (kDa)</th>
<th>Titer of Virus Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vac-TK^-</td>
<td>none</td>
<td>---</td>
<td>3 × 10^8 pfu/ml</td>
</tr>
<tr>
<td>Vac-EBNA1</td>
<td>BKRF1</td>
<td>60-85 kDa</td>
<td>2 × 10^9 pfu/ml</td>
</tr>
<tr>
<td>Vac-EBNA2A</td>
<td>BYRF1</td>
<td>~ 90 kDa</td>
<td>3 × 10^8 pfu/ml</td>
</tr>
<tr>
<td>Vac-EBNA3A</td>
<td>BLRF3/BERF1</td>
<td>~ 145 kDa</td>
<td>6 × 10^8 pfu/ml</td>
</tr>
<tr>
<td>Vac-EBNA3B</td>
<td>BERF2a/2b</td>
<td>~ 165 kDa</td>
<td>2 × 10^9 pfu/ml</td>
</tr>
<tr>
<td>Vac-EBNA3C</td>
<td>BERF3/4</td>
<td>~ 155 kDa</td>
<td>4 × 10^8 pfu/ml</td>
</tr>
<tr>
<td>Vac-EBNALP</td>
<td>BamHI-W1/W2</td>
<td>20-30 kDa</td>
<td>1 × 10^9 pfu/ml</td>
</tr>
<tr>
<td></td>
<td>BamHI-Y1/Y2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vac-LMP1</td>
<td>BNLF1</td>
<td>63 kDa</td>
<td>3 × 10^8 pfu/ml</td>
</tr>
<tr>
<td>Vac-LMP2A</td>
<td>U5-&gt;TR-&gt;U1</td>
<td>54 kDa</td>
<td>3 × 10^9 pfu/ml</td>
</tr>
<tr>
<td>Vac-gp350</td>
<td>BLLF1</td>
<td>350/220 kDa</td>
<td>3 × 10^9 pfu/ml</td>
</tr>
</tbody>
</table>
Table 4. Monoclonal antibodies and corresponding antigen specificities.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cluster of Differentiation</th>
<th>Reactivity of mAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-4</td>
<td>CD3</td>
<td>Human T cells; TCR</td>
</tr>
<tr>
<td>Leu-3a</td>
<td>CD4</td>
<td>Human, Helper T cells</td>
</tr>
<tr>
<td>OKT1</td>
<td>CD5</td>
<td>Pan T cell marker</td>
</tr>
<tr>
<td>Leu2a</td>
<td>CD8</td>
<td>Cytotoxic T Cells</td>
</tr>
<tr>
<td>Leu-15, Mo-1</td>
<td>CD11b</td>
<td>Human CR3; activated/memory CTL</td>
</tr>
<tr>
<td>Leu-11b</td>
<td>CD16</td>
<td>FcγRIII; NK cells</td>
</tr>
<tr>
<td>Leu-12</td>
<td>CD19</td>
<td>Pan B cell marker</td>
</tr>
<tr>
<td>Leu-16</td>
<td>CD20</td>
<td>Pan B cell marker</td>
</tr>
<tr>
<td>Leu-20</td>
<td>CD23</td>
<td>Activated B cells</td>
</tr>
<tr>
<td>Leu-18</td>
<td>CD45RA</td>
<td>Naive T cells</td>
</tr>
<tr>
<td>Leu-45RO</td>
<td>CD45RO</td>
<td>Memory T cells</td>
</tr>
<tr>
<td>Leu-19</td>
<td>CD56</td>
<td>NK cells, CTL subsets</td>
</tr>
<tr>
<td>Leu-7</td>
<td>CD57</td>
<td>T Cell / NK cell subsets</td>
</tr>
<tr>
<td>BBM.1</td>
<td>none</td>
<td>β2-microglobulin</td>
</tr>
<tr>
<td>W6/32</td>
<td>none</td>
<td>β2-microglobulin</td>
</tr>
<tr>
<td>BB7.7</td>
<td>none</td>
<td>β2-microglobulin</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>none</td>
<td>HLA class II molecules</td>
</tr>
<tr>
<td>72A1</td>
<td>none</td>
<td>EBV envelope gp350</td>
</tr>
<tr>
<td>S12</td>
<td>none</td>
<td>EBV LMP-1</td>
</tr>
<tr>
<td>TCR α/β-1</td>
<td>none</td>
<td>α/β+ T cells</td>
</tr>
<tr>
<td>TCR γδ-1</td>
<td>none</td>
<td>γδ+ T cells</td>
</tr>
</tbody>
</table>
Table 5. Activation Marker Expression on CD8+ T Cells from IM Patients.

<table>
<thead>
<tr>
<th>Acute IM Patient</th>
<th>% CD8+/DR+ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRIBR</td>
<td>44.2%</td>
</tr>
<tr>
<td>BUSRO</td>
<td>37.9%</td>
</tr>
<tr>
<td>GUZTH</td>
<td>72.6%</td>
</tr>
<tr>
<td>KAYSE</td>
<td>40.3%</td>
</tr>
<tr>
<td>CONMA</td>
<td>27.7%</td>
</tr>
<tr>
<td>CLAJO</td>
<td>41.7%</td>
</tr>
<tr>
<td>SCHSA</td>
<td>45.7%</td>
</tr>
<tr>
<td>NUFMA</td>
<td>43.7%</td>
</tr>
<tr>
<td>BECTE</td>
<td>69.2%</td>
</tr>
<tr>
<td>MINER</td>
<td>50.3%</td>
</tr>
<tr>
<td>GAFCH</td>
<td>20.8%</td>
</tr>
<tr>
<td>EXLZA</td>
<td>49.0%</td>
</tr>
<tr>
<td>MUSPA</td>
<td>39.4%</td>
</tr>
<tr>
<td>HESJA</td>
<td>28.9%</td>
</tr>
<tr>
<td>HEVJE</td>
<td>36.5%</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>43.2% +/- 13.8</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Healthy Controls</th>
<th>% CD8+/DR+ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>DELSA</td>
<td>9.1%</td>
</tr>
<tr>
<td>PTABA</td>
<td>10.8%</td>
</tr>
<tr>
<td>BEABR</td>
<td>10.1%</td>
</tr>
<tr>
<td>GREKI</td>
<td>5.7%</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>8.9% +/- 2.3%</strong></td>
</tr>
</tbody>
</table>
Table 6. Summary of Primary vs Secondary EBV-Specific CTL Responses.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Primary Response</th>
<th>Memory Response</th>
<th>Class-I Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliar</td>
<td>n.d.</td>
<td>EBNA 2A</td>
<td>A26,A31,B35,B38</td>
</tr>
<tr>
<td>Allba</td>
<td>EBNA 3A, 3C, LP</td>
<td>EBNA 1, 3A, 3C,Lp</td>
<td>A1,A28,B8,B40</td>
</tr>
<tr>
<td>Arach</td>
<td>EBNA 3B, 3C</td>
<td>n.d.</td>
<td>A3,A24,B7,B35</td>
</tr>
<tr>
<td>Aumre</td>
<td>LMP 1, EBNA 3C</td>
<td>EBNA 2A</td>
<td>A2,A30,B27,B40</td>
</tr>
<tr>
<td>Belch</td>
<td>EBNA 3A, 3B</td>
<td>EBNA 3B</td>
<td>A2,A34,B51,B53</td>
</tr>
<tr>
<td>Belki</td>
<td>EBNA 2A, 3C</td>
<td>EBNA 3A, 3B</td>
<td>A1,A3,B35,B49</td>
</tr>
<tr>
<td>Blabo</td>
<td>EBNA1,3A,3B,LMP1,gp350</td>
<td>EBNA 3B, 3A, 3C</td>
<td>A3,A32,B14,B40</td>
</tr>
<tr>
<td>Blabr</td>
<td>n.d.</td>
<td>LMP 1</td>
<td>A1,A28,B27,B40</td>
</tr>
<tr>
<td>Borth</td>
<td>EBNA 1</td>
<td>EBNA LP</td>
<td>A3, B18, B39</td>
</tr>
<tr>
<td>Busjo</td>
<td>EBNA 3B, 3C</td>
<td>EBNA 3C, 2A, 3A</td>
<td>A1,A3,B7,B40</td>
</tr>
<tr>
<td>Clima</td>
<td>EBNA 3C</td>
<td>n.d.</td>
<td>A2,B13,B18</td>
</tr>
<tr>
<td>Conja</td>
<td>gp350</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Corti</td>
<td>EBNA 1, 2B, 3C, gp350</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Croma</td>
<td>n.d.</td>
<td>EBNA 3A</td>
<td>A3,A24,B7,B62</td>
</tr>
<tr>
<td>Delsa</td>
<td>n.d.</td>
<td>EBNA 3A, 3B</td>
<td>A11,A34,B44,B62</td>
</tr>
<tr>
<td>Dowda</td>
<td>EBNA 1, 3A, LP</td>
<td>n.d.</td>
<td>A24,A28,B14,B39</td>
</tr>
<tr>
<td>Frije</td>
<td>EBNA 3C, LMP 2A</td>
<td>EBNA 3A, 3B, 3C</td>
<td>A2,A24,B44,B55</td>
</tr>
<tr>
<td>Gowad</td>
<td>EBNA 2A, 3A, 3B LMP2A</td>
<td>EBNA 3A, 3B, 3C, LMP2A</td>
<td>A2,A2,B40,B62</td>
</tr>
<tr>
<td>Halke</td>
<td>EBNA 3B</td>
<td>n.d.</td>
<td>A28, B41, B40</td>
</tr>
<tr>
<td>Hesru</td>
<td>n.d.</td>
<td>EBNA 3A, 3B</td>
<td>A1,A11,B8,B55</td>
</tr>
<tr>
<td>Kalma</td>
<td>EBNA 2A, 3C</td>
<td>n.d.</td>
<td>A2,A29,B39,B62</td>
</tr>
<tr>
<td>Kowre</td>
<td>EBNA LP</td>
<td>n.d.</td>
<td>A2,A3,B7,B44</td>
</tr>
<tr>
<td>Lanna</td>
<td>EBNA 1, 2A, 3B</td>
<td>n.d.</td>
<td>A1,A25,B8,B18</td>
</tr>
<tr>
<td>Levpa</td>
<td>EBNA 3B</td>
<td>EBNA 3A, 3B, 3C</td>
<td>A11,A30,B52</td>
</tr>
<tr>
<td>Levro</td>
<td>EBNA 2A, 3A, 3B, 3C, LP</td>
<td>EBNA 3C</td>
<td>A1,A24,B7,B38</td>
</tr>
<tr>
<td>Mahch</td>
<td>EBNA 3C</td>
<td>EBNA 3C</td>
<td>A1,A3,B8,B14</td>
</tr>
<tr>
<td>Marro</td>
<td>EBNA 3A, 3C</td>
<td>EBNA 3A</td>
<td>A3,A32,B8,B40</td>
</tr>
<tr>
<td>Marth</td>
<td>EBNA 3A, gp350</td>
<td>EBNA 3A, 3C</td>
<td>A1,A2,B7,B45</td>
</tr>
<tr>
<td>Mazma</td>
<td>EBNA 3C, LMP 2A</td>
<td>LMP 2A</td>
<td>A23,A24,B49</td>
</tr>
<tr>
<td>Mckna</td>
<td>EBNA 3A, LP</td>
<td>EBNA 3A</td>
<td>A1, B8, B14</td>
</tr>
<tr>
<td>Nicdo</td>
<td>EBNA 3A</td>
<td>EBNA 3A, 3C</td>
<td>A1,A28,B8,B14</td>
</tr>
<tr>
<td>Ptaba</td>
<td>n.d.</td>
<td>EBNA 3B, 3C</td>
<td>A2,A26,B13,B38</td>
</tr>
<tr>
<td>Ronch</td>
<td>EBNA 3A, 3C, gp350</td>
<td>EBNA 3C, LP</td>
<td>A2,A3,B7,B13</td>
</tr>
<tr>
<td>Saran</td>
<td>EBNA 3C, LMP 1</td>
<td>EBNA 3C</td>
<td>A24,A28,B44,B51</td>
</tr>
<tr>
<td>Shech</td>
<td>EBNA 3C</td>
<td>EBNA 3C</td>
<td>A2,A24,B7,B44</td>
</tr>
<tr>
<td>Stuje</td>
<td>EBNA 3A</td>
<td>EBNA2,3A,3B,3C,LMP2A</td>
<td>A3, B7,B55</td>
</tr>
<tr>
<td>Suljo</td>
<td>n.d.</td>
<td>EBNA 3B, 3C</td>
<td>A29,A32,B40,B44</td>
</tr>
<tr>
<td>Tayo</td>
<td>EBNA 3A, 1, LMP1</td>
<td>EBNA 3A, 3C</td>
<td>A3,A28,B7,B53</td>
</tr>
<tr>
<td>Trajo</td>
<td>EBNA 1, 3B, 3C, gp350</td>
<td>EBNA 3A, 3B</td>
<td>A11, A33, B35</td>
</tr>
<tr>
<td>Wilph</td>
<td>EBNA 3C</td>
<td>EBNA 3C, 3B</td>
<td>A2,A32,B27,B44</td>
</tr>
<tr>
<td>Yakme</td>
<td>EBNA3C,LP,LMP2A,gp350</td>
<td>n.d.</td>
<td>A2,A30,B13,B44</td>
</tr>
</tbody>
</table>

* Primary Responses equal composite of several assays.  * n.d. = not determined
Table 7. Acute IM patient CTL responses specific for EBNA-1:

<table>
<thead>
<tr>
<th>Targets</th>
<th>@Allba</th>
<th>Blabo</th>
<th>Borth</th>
<th>Corti</th>
<th>Dowda</th>
<th>Lanna</th>
<th>Tavjo</th>
<th>Trajo</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-Blasts</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Vac-TK</td>
<td>8.1</td>
<td>0.0</td>
<td>13.8</td>
<td>7.3</td>
<td>3.0</td>
<td>0.0</td>
<td>4.7</td>
<td>31.3</td>
</tr>
<tr>
<td>EBNA-1</td>
<td>24.2</td>
<td>14.0</td>
<td>24.5</td>
<td>24.9</td>
<td>13.8</td>
<td>16.0</td>
<td>24.4</td>
<td>44.7</td>
</tr>
<tr>
<td>EBNA-2</td>
<td>13.3</td>
<td>3.1</td>
<td>1.3</td>
<td>20.1</td>
<td>10.0</td>
<td>10.6</td>
<td>11.2</td>
<td>32.2</td>
</tr>
<tr>
<td>EBNA-3A</td>
<td>32.8</td>
<td>24.3</td>
<td>7.0</td>
<td>4.3</td>
<td>16.4</td>
<td>0.0</td>
<td>31.1</td>
<td>30.0</td>
</tr>
<tr>
<td>EBNA-3B</td>
<td>17.2</td>
<td>0.0</td>
<td>12.7</td>
<td>17.1</td>
<td>6.4</td>
<td>4.0</td>
<td>6.7</td>
<td>38.1</td>
</tr>
<tr>
<td>EBNA-3C</td>
<td>31.4</td>
<td>16.6</td>
<td>20.3</td>
<td>11.9</td>
<td>0.0</td>
<td>31.1</td>
<td>30.0</td>
<td></td>
</tr>
<tr>
<td>EBNA-LP</td>
<td>21.2</td>
<td>0.0</td>
<td>18.7</td>
<td>7.1</td>
<td>13.3</td>
<td>0.0</td>
<td>20.5</td>
<td>49.7</td>
</tr>
<tr>
<td>LMP-1</td>
<td>5.6</td>
<td>37.9</td>
<td>16.3</td>
<td>21.1</td>
<td>10.9</td>
<td>4.4</td>
<td>34.3</td>
<td>37.4</td>
</tr>
<tr>
<td>LMP-2</td>
<td>12.8</td>
<td>4.8</td>
<td>8.9</td>
<td>13.0</td>
<td>9.8</td>
<td>3.6</td>
<td>21.2</td>
<td>35.0</td>
</tr>
<tr>
<td>gp350</td>
<td>13.0</td>
<td>47.6</td>
<td>16.5</td>
<td>20.8</td>
<td>9.1</td>
<td>0.0</td>
<td>15.8</td>
<td>47.3</td>
</tr>
</tbody>
</table>

Values represent % specific lysis of Vac-infected targets minus background lysis of B blasts.

@ Only patient with EBNA-1-specific memory CTL response -> no EBNA1 primary response.

* Criteria used to determine significant target cell lysis:

- Spontaneous release of $^{51}$Cr from target cells < 25%.
- Lysis considered significant only if exceeded lysis of Vac-TK infected targets lysis by > 10%.
Table 8. Acute IM patient CTL responses specific for EBV-gp350:

Patients with significant* CTL responses directed against gp350

<table>
<thead>
<tr>
<th>Targets</th>
<th>Blabo</th>
<th>Conja</th>
<th>Corti</th>
<th>Marth</th>
<th>Ronch</th>
<th>Trajo</th>
<th>Yakme</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-Blasts</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Vac-TK</td>
<td>0.0</td>
<td>0.0</td>
<td>7.3</td>
<td>25.7</td>
<td>0.0</td>
<td>31.3</td>
<td>3.8</td>
</tr>
<tr>
<td>EBNA-1</td>
<td>14.0</td>
<td>0.0</td>
<td>24.9</td>
<td>34.8</td>
<td>2.3</td>
<td>44.7</td>
<td>4.6</td>
</tr>
<tr>
<td>EBNA-2</td>
<td>3.1</td>
<td>0.0</td>
<td>20.1</td>
<td>42.4</td>
<td>0.0</td>
<td>32.2</td>
<td>1.0</td>
</tr>
<tr>
<td>EBNA-3A</td>
<td>24.3</td>
<td>0.0</td>
<td>4.3</td>
<td>33.7</td>
<td>2.5</td>
<td>30.0</td>
<td>5.8</td>
</tr>
<tr>
<td>EBNA-3B</td>
<td>0.0</td>
<td>0.0</td>
<td>17.1</td>
<td>27.0</td>
<td>3.4</td>
<td>38.1</td>
<td>5.5</td>
</tr>
<tr>
<td>EBNA-3C</td>
<td>0.0</td>
<td>9.0</td>
<td>20.3</td>
<td>56.3</td>
<td>25.2</td>
<td>36.4</td>
<td>24.1</td>
</tr>
<tr>
<td>EBNA-LP</td>
<td>0.0</td>
<td>6.9</td>
<td>7.1</td>
<td>52.1</td>
<td>5.8</td>
<td>49.7</td>
<td>19.7</td>
</tr>
<tr>
<td>LMP-1</td>
<td>37.9</td>
<td>0.0</td>
<td>21.1</td>
<td>36.2</td>
<td>0.0</td>
<td>37.4</td>
<td>3.8</td>
</tr>
<tr>
<td>LMP-2</td>
<td>4.8</td>
<td>0.0</td>
<td>13.0</td>
<td>43.3</td>
<td>7.4</td>
<td>35.0</td>
<td>35.5</td>
</tr>
<tr>
<td>gp350</td>
<td>47.6</td>
<td>18.0</td>
<td>20.8</td>
<td>49.6</td>
<td>12.8</td>
<td>47.3</td>
<td>15.9</td>
</tr>
</tbody>
</table>

Values represent % specific lysis of Vac-infected targets minus background lysis of B blasts.

* Criteria used to determine significant target cell lysis:
- Spontaneous release of $^{51}$Cr from target cells < 25%.
- Lysis considered significant only if exceeded lysis of Vac-TK infected targets lysis by > 10%.
Figure 1. *EBV strain typing by DNA-PCR amplification of B cells and saliva from acute infectious mononucleosis patients.*

Lanes 1-4 contain PCR-amplified product from AIM patient B cells. Lanes 5-8 contain PCR-amplified product from AIM patient saliva. Lane 9 contains PCR-amplified product from the EBV type-1 infected line; Namalwa. Lane 10 contains PCR-amplified product from the EBV type-2 infected line; jijoye. Blot (a) was hybridized with the EBV type-1 specific probe and blot (b) was hybridized with the EBV type-2 specific probe.
Fig. 2a.
Separation of T- and B-lymphocyte subsets via SRBC-rosette selection.

Fig 2b. T- and B-lymphocyte enrichment via AET-SRBC rosette selection.

Rosette positive cells, approx. 90% T cells, used to establish CTL (effector).
Rosette negative cells, approx. 80% B cells, used to generate B-blast (targets).
a. EBV (-) T Cell Line (CEM).
b. B-Lymphoblastoid Cell Line (BLCL).
c. Vac-EBNA1 infected fibroblasts.
d. Vac-EBNA2a infected fibroblasts.
e. Vac-EBNA3a infected fibroblasts.
f. Vac-EBNA3b infected fibroblasts.
g. Vac-EBNA3c infected fibroblasts.
h. Vac-EBNALP infected fibroblasts.
a. Vac-TK infected human fibroblasts.
b. B lymphoblastoid cell line.
c. Vac-LMP1 + fibroblasts (5hrs).
d. Vac-LMP1 + fibroblasts (8hrs).
e. Vac-LMP1 + fibroblasts (10hrs).
f. Vac-LMP1 + fibroblasts (12hrs).
g. Vac-TK infected fibroblasts.
h. Vac-gp350 infected fibroblasts.
Fig 7. Activation marker expression on CD8+ T cells in acute IM patients.

Peripheral blood mononuclear cells were isolated from acute IM patients (Busro, Kayse and Guzth), stained with fluorochrome conjugated antibodies specific for CD8, HLA-DR, CD11b and subjected FACS analysis. Peripheral blood from acute IM patients contain large numbers of triple positive cells (quadrant 2) which are absent from blood samples derived from healthy control donors. The CD8+/HLA-DR+/CD11b+ cells represent activated, virus-specific, cytotoxic-T-cells which mediate lysis of EBV-infected B cells.
Peripheral blood samples were obtained from acute IM patients on sequential days in order to follow changes in cell surface marker expression. PBMC were stained with fluorochrome conjugated mAb specific for CD8, HLA-DR and CD11b prior to FACS analysis. Representative results shown here demonstrate a large population of CD8+/HLA-DR+/CD11b+ cells (quadrant 2) present early in the course of infection (day 1), which subsequently lose HLA-DR expression over a period of several weeks (50% reduction/week), but which retain high levels of CD11b expression for many months. These CD8+/HLA-DR-/CD11b+ cells are thought to represent long lived memory CTL which provide life long EBV immunity (also see fig 11).
Fig. 9. Kinetics of HLA-DR expression on CD8+ T-cells during acute IM.

PBMC from acute IM patients were stained with fluorochrome conjugated mAb specific for CD8, HLA-DR and CD57 prior to FACS analysis. Blood samples drawn at the time of initial patient evaluation (day 1) contain predominantly CD8+/HLA-DR+/CD57- cells (quadrant 1). As the acute phase of illness resolves, the percentage of CD8+/HLA-DR+ cells (activated CTL) is reduced (day 8), while the number of CD8+/HLA-DR-/CD11b+ (memory CTL) rises (see figures 8 + 11).
PBMC from acute IM patients (n=3) were stained with fluorochrome conjugated mAb specific for CD8, HLA-DR and CD11b (Mo-1) prior to FACS analysis. High levels of triple positive cells present on day one decline with resolution of the acute phase of infection. The long lived CD8+/HLA-DR-/CD11b+ population likely represent memory CTL and are maintained at high levels in the peripheral blood for many months following infection.
Fig 11. Kinetics of HLA-DR vs CD11b (Mo-1) on CD8+ T-cells following acute IM.

Facs analysis was utilized to measure levels of HLA-DR and CD11b on CD8+ T cells following EBV infection. At 6 weeks post-infection, HLA-DR expression was significantly reduced, while CD11b expression remained maximally elevated. At 6 months post-infection, HLA-DR expression returned to baseline levels, while CD11b levels remained at twice the level of healthy controls.
Fig. 12a. Non-specific lysis of NK-sensitive targets (K562) mediated by unstimulated, whole PBMC from EBV-seropositive donor SULJO.

Fig. 12b. Bulk cultures of BLCL-stimulated PBMCs, not NK depleted, mediate lysis of NK-sensitive (K562) & EBV infected targets.

Fig. 12c. Bulk cultures of BLCL-stimulated PBMCs, depleted of NK cells, primarily lyse HLA-matched, EBV-infected targets.

Fig. 12d. EBV-specific CTL cannot be cultured from the peripheral blood of EBV-seronegative individuals.
Fig. 13a. EBV-specific memory CTL lyse B-blasts expressing individual EBV genes (EBNAs 3b+3c).

Fig. 13b. EBV-specific memory CTL lyse fibroblasts expressing individual EBV genes (LMP-1).

Fig. 13c. In-vitro restimulated EBV-specific memory CTL lyse B-blasts expressing individual EBV latent genes (EBNA 3a+3b).

Fig. 13d. In-vitro restimulated EBV-specific memory CTL lyse B-blasts expressing individual EBV latent genes (EBNA 3a+3b).
Fig. 14a. In-vivo activated CTL from acute IM patients preferentially lyse EBV-infected target cells.

Fig. 14b. SRBC-rosette(+) T cells from acute IM patients lyse both HLA-matched and HLA-unmatched target cells.

Fig. 14c. SRBC-rosette-selected NK-depleted CTL from acute IM patients preferentially lyse HLA-matched target cells.

Fig. 14d. The primary immune response to EBV includes virus-specific CTL which lyse allogeneic HLA-matched targets expressing individual EBV genes.
Fig. 15. Representative composite of primary EBV-specific CTL responses in acute JM patients.

In-vivo activated CD8+ effector-cells were prepared as described previously. Target cells consisted of autologous B-blasts infected with recombinant vaccinia virus constructs. Criteria for significant lysis includes spontaneous release of 51Cr < 25%, and specific lysis >10% above Vac-TK (control). Results: 90% of patient' CTL lyse targets expressing one of the EBNA3 proteins, with 60% recognizing EBNA-3C alone. Seven of 35 patient' CTL lysed targets expressing EBNA-1. A detailed summary is included in table 5.
Levro-B Blasts
Levro Vac-TK
Vac-EBNA 1
Vac-EBNA 2A
Vac-EBNA 3A
Vac-EBNA 3B
Vac-EBNA 3C
Vac-EBNA LP
Vac-LMP1
Vac-LMP2A
Vac-gp350
Levro BLCL

Target Cells

% Specific Lysis

Fig. 16a. Target cell lysis by in-vivo primed, EBV-specific CTL is blocked by mAb directed against HLA class-I molecules.

RM-B Blasts
RM-Vac-TK
Vac-EBNA 1
Vac-EBNA 2A
Vac-EBNA 3A
Vac-EBNA 3B
Vac-EBNA 3C
Vac-EBNA LP
Vac-LMP1
Vac-LMP2A
Vac-gp350
K562

Target Cells

% Specific Lysis

Fig. 16b. Target cell lysis by in-vivo primed, EBV-specific CTL is partially blocked by mAb directed against CD8.
Fig. 17. Surface marker phenotype of SRBC-rosette (+), NK-depleted PBMCs from acute IM patients.
Fig. 18a. In vitro expanded memory CTL from acute IM patient "Mckma" lyse targets expressing single EBV genes.

Fig. 18b. In vitro expanded memory CTL from acute IM patient "Marth" lyse targets expressing single EBV genes.

Fig. 18c. In vitro expanded memory CTL from acute IM patient "Blabo" lyse targets expressing single EBV genes.

Fig. 18d. In vitro expanded memory CTL from acute IM patient "Frije" lyse targets expressing single EBV genes.
Fig. 19a. **Primary** CTL response to EBV by acute IM patient "Marro".

Fig. 19b. **Memory** CTL response to EBV by acute IM patient "Marro".

Fig. 19c. **Primary** CTL response to EBV by acute IM patient "Tavjo".

Fig. 19d. **Memory** CTL response to EBV by acute IM patient "Tavjo".
Fig. 20a. Primary CTL response to EBV by acute IM patient "Alba".

Fig. 20b. Memory CTL response to EBV by Acute IM Patient "Alba".
Fig. 21. Primary vs Memory CTL Responses to Epstein-Barr virus.
Fig 22a. Target cell lysis by PBMC stimulated with Vac-EBNA1 infected B blasts.

Fig 22b. Target cell lysis by PBMC stimulated with Vac-EBNA1 infected BLCLs.
Fig 23. Target cell lysis by acute IM patient "Levro" PBMC stimulated with Vac-EBNA1/B cell blasts.
Fig 24a. Cold target competition of PBMC stimulated with Vac-EBNA1/B Blasts.

Fig 24b. Cold target competition of PBMC stimulated with Vac-EBNA1/B Blasts.
Fig 25. Surface marker phenotype of Vac-EBNA1 stimulated bulk CTL culture.
Fig 26. Surface marker phenotype of Vac-EBNA1 stimulated bulk CTL culture.
REFERENCES

Abbas, A. (personal communication).


of normal B cells latently infected with Epstein-Barr virus resembles Burkitt lymphoma cells in expressing EBNA-1 but not EBNA-2 or LMP1. J. Virol. 69, 3752-3758.


Hill, A., Bladen, R., Parish, C., and Mullbacher, A. (1992). Restimulated memory Tc cells have higher apparent
avidity of interaction with targets than primary virus-immune Tc cells as indicated by anti-CD8 blocking. Immunol. Cell Biol. 70, 259.


Hsu, D., Waal-Malefyt, R. d., Fiorentino, D., Dang, M., Vieira, P., Vries, J. d., Spits, H., Mosmann, T., and


Jones, M., Foster, L., Sheedy, T., and Griffin, B. (1984). The EB virus genome in Daudi Burkitt's lymphoma cells has a deletion similar to that observed in a non-transforming strain (P3HR-1) of the virus. EMBO Journal 3, 813-821.


of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. J. Exp. Med. 178, 1801-1806.


and transformation in vitro; LMP2A is therefore nonessential. J. Virol. 66, 6461-6469.


Virol. 65, 6826-6837.


Merl, S., Kloster, B., Moore, J., Hubbell, C., Tomar, R., Kalinowski, F. D., Planas, A., Erlich, G., Clark, D,


(EBV) genome after transfection of the EBV DNA fragment. J. Virol. 57, 1016-1022.


Wysokenski, D., and Yates, J. (1989). Multiple EBNA-1-binding sites are required to form an EBNA-1-dependent enhancer and to activate a minimal replicative origin within ori P of Epstein-Barr virus. J. Virol. 63, 2657-2666.


Young, L., Dawson, C., Clark, D., and al., e. (1988). Epstein-Barr virus gene expression in nasopharyngeal


