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Epstein Barr Virus

Methods and Protocols

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Epstein Barr Virus

Methods and Protocols

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Cover illustration: The picture is derived from the chapter “Functional Analysis of Exosomes Derived from EBV-Infected Cells” of this book, by Gulfaraz Khan and Pretty S. Philip.

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Foreword

The Epstein–Barr virus (EBV) was identified 49 years ago as the causative agent of a long time known disease, infectious mononucleosis (IM, glandular fever). The IM symptoms are not manifested by all individuals at the time of acquisition of the infection. It is highly important that the subclinical primary infection also leads to the virus carrier state for lifetime accompanied by efficient immunity. The "silent" infection is due to a particular strategy of the immune system, based on a finely tuned interaction between T and B lymphocytes. When EBV-carrying B lymphocytes acquire proliferating potential (thus malignant potential) in a well-defined differentiation window in vivo, their multiplication is controlled, and the threat of lymphoma development is eliminated. In vitro experiments show that EBV can induce B cell proliferation directly, without the contribution of additional factors, as demonstrated by the establishment of immortalized lines (LCLs, lymphoblastoid cell lines). It is worth mentioning that LCLs are not only essential tools in basic EBV research, but these lines were indispensable in human genetic studies as well. Using several further strategies EBV contributes to malignant transformation of lymphocytes and also of epithelial and mesenchymal cells. By triggering or perpetuating pathogenic processes, EBV may also play a role in the development of autoimmune syndromes including multiple sclerosis, systemic lupus erythematosus (SLE), and rheumatoid arthritis. The complex and versatile (non-pathogenic and pathogenic) EBV–human interactions are well known. This volume provides a good background for continued studies with details of molecular, immunological, and cell biological methods. Guidelines for the treatment of certain EBV-associated malignancies are also given. "Hot topics" of the field are overviewed in the introductory chapter by the *Editors*, Janos Minarovits (Szeged) and Hans Helmut Niller (Regensburg), followed by the chapters of experts who master the thoroughly described methods. These chapters are highly helpful for both basic and clinical scientists.

Stockholm, Sweden ***Eva Klein***

Preface

Fifty-two years ago, collaboration between a dedicated surgeon and basic researchers, who applied a combination of as diverse laboratory methods as in vitro cell culture and electron microscopy, paved the way to the discovery of Epstein–Barr virus (EBV), a human herpesvirus (reviewed by Epstein [1]). The detection of herpesvirus particles in Burkitt lymphoma (BL) cells supported the idea—suggested by Denis Burkitt—that an infectious agent may cause BL, a B cell lymphoma highly prevalent among children in Equatorial Africa. Ever since, newer and newer analytic methods were applied to the field of EBV research, shaping our current views as to the natural history of EBV and the pathogenesis of EBV-associated diseases (for a historical perspective, see Ref. [2]). Furthermore, BL was regarded "as a Rosetta stone for understanding the multistep carcinogenesis," i.e., observations such as EBV and *Plasmodium malariae* infection of children with endemic BL and the discovery of *c-myc* translocations in BL cells influenced thinking as to the development of other neoplasms, too [3]. De Thé et al. referred in their paper to a bilingual text, carved in three scripts into a rock stele that was found in the Nile Delta near the town of Rosetta (Rashid); that text—a new research tool, if you wish—was instrumental for the decipherment of Egyptian hieroglyphs by Jean-François Champollion, an achievement that allowed the translation of Ancient Egyptian texts and facilitated the understanding of Egyptian culture and civilization [4]. The data gained by the application of molecular biological methods combined with the applications of up-to-date immunological and cytogenetic methods as well as in vitro and in vivo models of EBV research may offer new solutions to unresolved problems of the EBV field. Some of the hot topics and alternative scenarios as to the viral life cycle, latency, and EBV-associated diseases are overviewed in Chapter 1 of this volume, in the light of the most recent findings, by Janos Minarovits and Hans Helmut Niller. This introductory chapter is followed by typical *Methods* chapters written by experts of the respective areas. Hans Helmut Niller and Georg Bauer give a description of the immunological and molecular methods used in EBV diagnostics (Chapter 2). It is not easy to investigate in vivo EBV infection of B lymphocytes and epithelial cells, the major cell types targeted by EBV. The use of in vitro models facilitates, however, the study of EBV–host cell interactions. Such models include the establishment of "immortalized" lymphoblastoid cell lines by EBV infection of human B lymphocytes, as presented by Noémi Nagy (Chapter 3), and the use of organotypic cultures for the analysis of EBV–epithelial cell interactions, as described thoroughly by Rachel M. Temple, Craig Meyers, and Clare E. Sample (Chapter 4). Identification of the interacting viral and cellular proteins is indispensable for the understanding of interrelationships between EBV and its host cells. In Chapter 5, Anna A. Georges and Lori Frappier guide us how to use affinity purification-mass spectroscopy methods for identifying protein–protein interactions in EBV-infected cells. Certain latent EBV proteins influence the nuclear architecture of host cells. Hans Knecht and Sabine May elaborated a spectacular method for the characterization—in three dimensions—of nuclear architecture. They demonstrate how to use 3D Telomere FISH (fluorescent in situ hybridization) to detect nuclear changes, including the alterations of chromosomal ends, induced by LMP1 (latent membrane protein

1, a viral oncoprotein) in Hodgkin lymphoma cells (Chapter 6). Next-generation sequencing methods allow the detailed analysis of transcript structure and abundance in EBV-infected cells. In Chapter 7, Tina O'Grady, Melody Baddoo, and Erik K. Flemington outline the use of high-throughput RNA sequencing for the analysis of EBV transcription and guide the reader regarding the application of informatics tools for the analysis and visualization of sequence data. Two other approaches, quantitative polymerase chain reaction (qPCR) following conventional RNA isolation and nuclear run-on assay that are suitable for the study of viral promoter activity, are described in Chapter 8 by Kálmán Szenthe and Ferenc Bánáti. In addition to mRNAs, the EBV genome also encodes nontranslated viral microRNAs that modulate the level of both viral and cellular mRNAs and proteins. Furthermore, EBV latency products may alter the level of cellular microRNAs as well. Accordingly, the analysis of viral and cellular microRNAs, the topic of Chapter 9 written by Rebecca L. Skalsky, is of primary importance in the characterization of EBV-infected cells. MicroRNAs and other biomolecules are packaged into exosomes, small lipid vesicles involved in intercellular communication. EBV-infected cells use exosomes for information transfer as well, and the methods for exosome isolation and characterization as well as their functional analysis are detailed in Chapters 10 and 11 by Gulfaraz Khan and Waqar Ahmed, and Gulfaraz Khan and Pretty S. Philip, respectively. Regarding the methods used to study viral DNA, in Chapter 12 Ferenc Bánáti, Anita Koroknai, and Kálmán Szenthe describe how the clonality of a cell population carrying latent EBV episomes can be inferred from the "classical" terminal repeat analysis of the viral genome, whereas Chapter 13, by Kálmán Szenthe and Ferenc Bánáti, deals with the application of sequencing for the characterization of viral promoters and coding regions. Similarly to cellular promoters, the activity of latent and lytic EBV promoters is also regulated by the binding of viral and cellular regulatory proteins and by epigenetic mechanisms. In Chapter 14, Anja Godfrey, Sharada Ramasubramanyan, and Alison J. Sinclair demonstrate the use of ChIP-Seq method (chromatin precipitation coupled to DNA sequencing) for the analysis of Zta–DNA interactions. Zta, also called ZEBRA, is an immediate early protein switching on lytic (productive) EBV replication, and there are Zta binding sites both in the viral and in the cellular genome. Host cell phenotype-dependent deposition of epigenetic marks, including DNA methylation and histone modifications, determines the epigenotypes of latent EBV episomes and the activity of the latency promoters. In a detailed protocol, Daniel Salamon describes the use of bisulfite sequencing for the analysis of cytosine methylation in EBV DNA sequences and lists thoroughly the important steps and caveats of bisulfite modification and PCR amplification (Chapter 15), whereas in Chapter 16 Ferenc Bánáti and Kálmán Szenthe outline how chromatin immunoprecipitation using specific antibodies directed to distinct histone modifications can be applied for the characterization of viral epigenotypes. In vivo experimental models may help to gain insight into important aspects of the EBV life cycle and into the pathogenesis of EBV-associated diseases that are difficult to study in EBV-infected humans. In Chapter 17, Frank Heuts and Noemi Nagy describe how newborn immunodeficient mice transplanted with human hematopoietic stem cells can be used for the study of immune interactions that occur during EBV infection, whereas in Chapter 18 Ken-Ichi Imadome and Shigeyoshi Fujiwara provide detailed protocols for the preparation and EBV infection of humanized mice and for the monitoring of virological and immunological consequences of the infection. They also describe the development of EBV-associated lymphoproliferative disease in such mice.

Finally, Lauren P. McLaughlin, Stephen Gottschalk, Cliona M. Rooney, and Catherine M. Bollard describe, in Chapter 19, how immunological, virological, tissue culture, and molecular methods can be combined to yield GMP (Good Manufacturing Practice)-compliant EBV-specific T cells for the immunotherapy of EBV-associated post-transplant lymphoproliferative disease (PTLD).

Budapest, Hungary ***Janos Minarovits***
Regensburg, Germany ***Hans Helmut Niller***

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Contents

Preface . . . . *vii*
Contributors . . . . *xiii*

1 Current Trends and Alternative Scenarios in EBV Research 1
Janos Minarovits and Hans Helmut Niller

2 Epstein-Barr Virus: Clinical Diagnostics 33
Hans-Helmut Niller and Georg Bauer

3 Establishment of EBV-Infected Lymphoblastoid Cell Lines 57
Noemi Nagy

4 Generation and Infection of Organotypic Cultures with Epstein–Barr Virus. . . 65
Rachel M. Temple, Craig Meyers, and Clare E. Sample

5 Affinity Purification–Mass Spectroscopy Methods for Identifying Epstein–Barr Virus–Host Interactions 79
Anna A. Georges and Lori Frappier

6 The Use of 3D Telomere FISH for the Characterization of the Nuclear Architecture in EBV-Positive Hodgkin's Lymphoma 93
Hans Knecht and Sabine Mai

7 Analysis of EBV Transcription Using High-Throughput RNA Sequencing . . . 105
Tina O'Grady, Melody Baddoo, and Erik K. Flemington

8 Analysis of Viral Promoter Usage in EBV-Infected Cell Lines: A Comparison of qPCR Following Conventional RNA Isolation and Nuclear Run-On Assay 123
Kalman Szenthe and Ferenc Banati

9 Analysis of Viral and Cellular MicroRNAs in EBV-Infected Cells 133
Rebecca L. Skalsky

10 Isolation and Characterization of Exosomes Released by EBV-Immortalized Cells 147
Gulfaraz Khan and Waqar Ahmed

11 Functional Analysis of Exosomes Derived from EBV-Infected Cells 159
Gulfaraz Khan and Pretty S. Philip

12 Terminal Repeat Analysis of EBV Genomes 169
Ferenc Bánáti, Anita Koroknai, and Kálmán Szenthe

13 Characterization of EBV Promoters and Coding Regions by Sequencing PCR-Amplified DNA Fragments 179
Kalman Szenthe and Ferenc Bánáti

14 The Use of Chromatin Precipitation Coupled to DNA Sequencing (ChIP-Seq) for the Analysis of Zta Binding to the Human and EBV Genome 191
Anja Godfrey, Sharada Ramasubramanyan, and Alison J. Sinclair

15 Analysis of Viral Epigenotypes Using Bisulfite Sequencing: A Detailed Protocol for the Crucial Bisulfite Modification and PCR Amplification Steps 207
Daniel Salamon

16 Analysis of Viral Epigenotypes Using Chromatin Immunoprecipitation 215
Ferenc Bánáti and Kálmán Szenthe

17 Mice with Reconstituted Human Immune System Components as a Tool to Study Immune Cell Interactions in EBV Infection 229
Frank Heuts and Noemi Nagy

18 Generation and Analysis of Humanized Mouse Model of EBV Infection 241
Ken-Ichi Imadome and Shigeyoshi Fujiwara

19 EBV-Directed T Cell Therapeutics for EBV-Associated Lymphomas 255
Lauren P. McLaughlin, Stephen Gottschalk, Cliona M. Rooney, and Catherine M. Bollard

Index *267*

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Chapter 1

Current Trends and Alternative Scenarios in EBV Research

Janos Minarovits and Hans Helmut Niller

Abstract

Epstein-Barr virus (EBV) infection is associated with several distinct hematological and epithelial malignancies, e.g., Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma, gastric carcinoma, and others. The association with several malignant tumors of local and worldwide distribution makes EBV one of the most important tumor viruses. Furthermore, because EBV can cause posttransplant lymphoproliferative disease, transplant medicine has to deal with EBV as a major pathogenic virus second only to cytomegalovirus. In this review, we summarize briefly the natural history of EBV infection and outline some of the recent advances in the pathogenesis of the major EBV-associated neoplasms. We present alternative scenarios and discuss them in the light of most recent experimental data. Emerging research areas including EBV-induced patho-epigenetic alterations in host cells and the putative role of exosome-mediated information transfer in disease development are also within the scope of this review. This book contains an in-depth description of a series of modern methodologies used in EBV research. In this introductory chapter, we thoroughly refer to the applications of these methods and demonstrate how they contributed to the understanding of EBV-host cell interactions. The data gathered using recent technological advancements in molecular biology and immunology as well as the application of sophisticated in vitro and in vivo experimental models certainly provided deep and novel insights into the pathogenetic mechanisms of EBV infection and EBV-associated tumorigenesis. Furthermore, the development of adoptive T cell immunotherapy has provided a novel approach to the therapy of viral disease in transplant medicine and hematology.

Key words Burkitt lymphoma, Exosome, EBV latency, Lytic viral replication, Hodgkin lymphoma, Latent membrane protein 1 (LMP1), Mass spectrometry, Patho-epigenetics, RNA-seq, Telomere, Tumorigenesis, In vivo experimental models, Adoptive T cell immunotherapy

1 Introduction

The virus particles of Epstein-Barr virus (EBV), a human herpesvirus infecting the majority of the population, were discovered by electron microscopy 52 years ago, in the suspension culture of a Burkitt lymphoma-derived cell line [1]. Last year, in connection with the 50th anniversary of the discovery, a series of reviews appeared, dealing with the natural history of EBV, the first human "tumor virus," and the many diseases associated with EBV infection [2, 3]. For this reason, here we wish to give only a brief summary of the basic facts as to the course of EBV infection and its

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pathologic consequences and focus, in the light of the most recent publications, on some of the emerging new topics and unresolved questions of the field.

2 Epstein-Barr Virus: Basic Facts

Epstein-Barr virus (EBV, also known as human herpesvirus 4) belongs to the *Lymphocryptovirus* genus within the *Gammaherpesvirinae* subfamily of the family *Herpesviridae*. As the name *Lymphocryptovirus* reflects, EBV infects lymphoid cells and "hides," i.e., establishes latency in the B lymphocyte compartment. Latency is a remarkable feature of herpesviruses that are capable to maintain their genomes in host cells for an extended period in the absence of virion production. Similarly to other herpesviruses, the EBV genome packaged into the virion is also a linear double-stranded DNA molecule surrounded by a membrane-coated icosahedral capsid. The prototype B95-8 EBV genome was the first completely sequenced herpesvirus genome [4], and, recently, with the advent of next-generation sequencing, the complete genomes of a series of EBV strains from multiple tumor cell lines, tumor types, and normal infection were determined [5–7] (reviewed by [8]).

EBV is associated with a series of malignant tumors including Burkitt lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin lymphoma (HL), posttransplant lymphoproliferative disease (PTLD), T/NK cell lymphomas, gastric carcinoma (EBVaGC), AIDS-associated lymphoma, leiomyosarcoma, and others [9–11]. The expression pattern of viral latent genes in EBV-associated tumor biopsies, in tumor-derived cell lines, and in EBV-infected B cell cultures largely depends on the tumor cell type and on the B cell differentiation status. For a detailed description of the different latent gene expression patterns, we refer to recent reviews [11, 12]. Traditionally, three latency classes I to III and sometimes latency type 0 ("zero") are discerned, accompanied by a few latency types which are situated in between the classical three latency types. In short, latency type 0 in memory B cells is rather restricted to the expression of the EBER RNAs only, possibly accompanied by expression of the BART RNAs or LMP2A in addition, and occasional EBNA1 expression, which is needed for viral genome maintenance when a memory cell is dividing. Latency type I is found in BL cells, with the expression of the EBERs, EBNA1 by transcription from the Q promoter (Qp), and the BART mRNAs and microRNAs. Additional variant expression of latent membrane protein (LMP) 2A is found in EBVaGC. Latency type II, with the additional expression of the LMPs 1 and 2, is found in Hodgkin lymphoma and in nasopharyngeal carcinoma (NPC) with a variable expression of LMP1. The expression of all viral latent gene products including the BHRF1 microRNAs is found in EBV-

transformed lymphoblastoid cell lines (LCL), in most early-onset PTLD cases and also in leiomyosarcomas of immune-suppressed patients. In this case, all EBNA proteins are expressed by transcription from the C promoter. This gene expression pattern has been termed "latency type III" or "Cp-on latency." This condition is also referred to as "the growth program" [13].

Besides the classical latent gene products, there are also specific gene products normally attributed to the lytic viral replication cycle which are regularly expressed in tumor tissue. For example, in NPC and EBVaGC, expression of the BARF1 protein is regularly found [14–16] (reviewed by [17]). However, there is more to be found among lytic genes which are regularly expressed in tumor tissue. Whole genome analysis of RNA-seq data has the power to uncover previously overlooked or hidden gene expression patterns, novel genes, and splice junctions, but also the contamination of cell lines [18–24]. A recent analysis of RNA-seq data of a series of EBVaGC revealed the expression of the BNLF2A reading frame in nearly half of all EBVaGC [25, 26]. BNLF2A codes for an inhibitor of MHC class I-TAP-mediated antigenic peptide transport and thereby provides immune evasion properties to latently infected tumor cells [27]. Furthermore, an RNA-seq analysis of NPC tissue and the NPC cell line C666.1 showed diverse latency gene expression patterns besides latency type II and a considerable amount of lytic regulatory gene expression [28].

Besides its classical role as a viral transcription and replication factor binding to oriP, latent protein EBNA1 is also expressed in the lytic viral infection mode and has manifold cellular impact. Affinity purification coupled with mass spectrometry (AP-MS) and tandem affinity purification (TAP-tagging) approaches have successfully been used to uncover the interaction of viral proteins with its cellular binding partners [29]. Through its direct interaction with the ubiquitin-specific protease (USP) 7 (also termed HAUSP), EBNA1 interferes with p53 and Mdm2 binding to the same binding pocket on USP7 in vitro [30, 31]. Thus, EBNA1 may contribute antiapoptotic functions to tumorigenesis in NPC or EBVaGC by lowering p53 levels in response to DNA damage [32, 33]. By disrupting PML nuclear bodies, similar to other herpesviruses, EBNA1 may contribute to the reactivation of the lytic viral replication cycle [34]. The situation is complex, however, because in epithelial cell lines EBNA1 upregulated a set of cellular microRNAs, including let-7a that represses EBV reactivation [35].

PML disruption through EBNA1 is mediated by the cellular kinase CK2 [30, 36]. Because the loss of PML proteins or nuclear bodies also impairs apoptosis [37], it is interesting that latent EBNA1 has been found to induce the loss of PML proteins in NPC and EBVaGC cells. Thereby, EBNA1 may contribute additional antiapoptotic functions to the establishment of EBV-associated malignancies [32, 33]. A further interaction partner of